Winter 2017

BINK DOMAIN FUNCTIONAL CHARACTERIZATION IN THE REGULATION OF BIOLUMINESCENCE IN VIBRIO FISCHERI

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THESIS

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Master of Science
in
Biochemistry

December, 2017

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On October 13th, 2017

Original approval signatures are on file with the University of New Hampshire Graduate School.

ACKNOWLEDGEMENTS

I would like to extend a thank you to my advisor Dr. Cheryl Whistler for mentoring and guiding me along my journey. My committee members Dr. Estelle Hrabak and Dr. Krisztina Varga for thoughtful input and direction of this thesis. Randi Foxall for all her guidance, thoughtful input, and mentorship as I became acclimated to the laboratory. Dr. Karen Visick for her insightful discussions and providing her strains, especially the KV7860 strain. Dr. Sabrina Pankey, Dr. Megan Thompson, and Randi Foxall for their assistance in learning R for graphing and statistical analysis. Jennifer Calawa, Christopher Nordyke, and Abigail Mark for proofreading. To my undergraduate researchers Nicole Clark and Ashley Gagnon for their hard work and dedication. To my family and friends for believing in me and supporting me. Lastly, I could not have done this without the amazing friendship of the Whistler laboratory, for not only making this a warm, inviting environment full of laughter, but an environment where we all helped each other accomplish our goals.

This research was funded by National Science Foundation Grant #1258099 and the University of New Hampshire Graduate School Summer Teaching Assistant Fellowship 2015.

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ABSTRACT

BINK DOMAIN FUNCTIONAL CHARACTERIZATION IN THE REGULATION OF BIOLUMINESCENCE IN *VIBRIO FISCHERI*

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Prokaryotes encode a remarkable ability to adapt to niches by sensing environmental cues through signal transduction systems (STSs). Typical STS proteins interact through a phosphorylation relay between histidine (His) and aspartate (Asp) residues within modular domains on sensory kinase and response regulator (RR) proteins to elicit cellular responses. A single point mutation in the sensor kinase BinK (BinK1 R537C) conferred an outstanding ability for the non-native *V. fischeri* strain MJ11 to successfully colonize *Euprymna scolopes* by affecting multiple symbiotic phenotypes including luminescence activation. However, the role of BinK in luminescence, the interacting partners, and functional mechanism are unknown. We hypothesized that BinK interacts upstream of an orphaned RR and acts as a canonical sensor kinase using a C-terminal receiver (REC) domain to activate luminescence. Heterologous multi-copy expression of BinK in native *V. fischeri* strain ES114 demonstrated that BinK does not utilize an orphan RR, but instead interfaces with the LuxU-LuxO node to activate luminescence. Additionally, BinK with a truncated REC domain and a REC domain with an aspartate – alanine substitution abolished luminescence activation where the level of light emitted matched the level of light emitted by a strain harboring the empty vector plasmid, suggesting BinK activates

luminescence in a REC-dependent manner using the conserved Asp residue for suspected phosphatase / dephosphorylation activity. Elimination of the kinase / auto-phosphorylation activity of the HisKA domain by incorporating a histidine – glutamine substitution did not alter BinK luminescence activation. Though these findings demonstrate one mechanism by which BinK activates luminescence, it is still not clear how the evolved *binK1* R537C mutation in the HATPase catalytic domain, a domain important in kinase function, influences REC-dependent dephosphorylation. By using multi-copy expression, BinK1 reduces luminescence and increases *qrr1* expression, and like BinK, works in a REC-dependent manner. These data suggest that one way BinK1 conferred the jump to symbiosis was through reduced or altered function. Furthermore, this mutation unveiled BinK as another potential regulator in bioluminescence where it is poised to work in a manner similar to quorum sensing activators AinR and LuxQ to activate luminescence.

I. INTRODUCTION

I.a. Signaling transduction systems

Signaling transduction systems (STS) utilize phosphorylation transfer between conserved phosphorelay domains on sensor and response regulator proteins to sense and respond to environmental changes and elicit cellular responses. Multi-domain protein structure facilitates phosphate transfer (Fig. 1). The generalized mechanism in a classical two-component system (Stock et al., 2000; Zschiedrich et al., 2016) begins with a series of phosphate transfers where a histidine kinase (HK) accepts an external signal via its sensory domain, triggering cleavage of a γ-phosphate from an ATP molecule by its HATPase_C (Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase) domain**.** The newly cleaved phosphate is transferred to a conserved histidine residue (His~P) within the kinase's dimerization and histidine containing (Dhp) domain, also annotated as a HisKA domain. Once the HK has autophosphorylated, the receiver (REC) domain of a response regulator protein (RR) catalyzes the transfer of this phosphate to its conserved aspartate residue (Asp~P). Upon this final phosphorylation, the RR elicits a cellular response via its effector domain, typically affecting gene regulation and expression, where the phosphate is either removed from the HisKA domain or the REC domain through phosphatase activity, resetting the signaling cascade. More complex STS pathways involve hybrid HKs with an elongated pathway of phosphorylation flow. Hybrid HKs contain a second phosphorylation site in the form of a fused REC domain and also utilize an intermediate histidine

phosphotransferase (HPt) protein or HPt domain that acts as a relay between the hybrid HK and RR proteins (Fig. 1B).

Hybrid and non-hybrid sensors primarily function in a kinase-dependent manner where signal transduction is initiated via autophosphorylation from ATP, but these sensors also can switch between kinase (forward phosphate flow) and phosphatase (reverse phosphate flow) activity**.** For instance, the REC domain of the hybrid HK protein LuxN in *Vibrio harveyi* exhibits constitutive phosphatase activity, and this activity is amplified upon quorum-signal activation, which turns off kinase activity but retains phosphatase activity (Freeman et al., 2000). A more in-depth look at other hybrid HKs revealed that the hydrolysis of the Asp~P on the REC domain due to the REC domain's phosphatase activity is preferred over the other His~Ps, including the transfer to an HPt domain. Furthermore, replacement of the Asp residue with an alanine enhanced the autophosphorylation of the His residue within the HisKA domain (Kinoshita-Kikuta et al., 2015). The HisKA domains of non-hybrid HK systems also exhibit phosphatase activity but usually require outside molecular aid. For example, in the NtrB/NtrC system, the REC domain of the RR NtrC uses autophosphatase activity, while the HK NtrB dephosphorylates NtrC via a helper regulator protein P_{II} (Keener & Kustu, 1988). In the HK-RR pair PhoQ/P, abolishing phosphatase in PhoQ significantly alters cellular physiology for many generations and carries a fitness cost (Ram & Goulian, 2013). This dual kinase/phosphatase activity is integral in temporal regulation of STS functionality whereby altering function drastically alters cell regulation and fitness.

Simpler STS are ubiquitous across prokaryotes and typically insulated wherein only paired partner proteins specific to that STS interact. Common pathways include but are not limited to: osmolarity regulation by EnvZ/OmpR (Forst & Roberts, 1994; Russo & Silhavy,

1991), nitrogen regulation by NtrB/NtrC (Taylor et al., 2015), and chemotaxis through CheA/CheY (Bren & Eisenbach, 2000; Parkinson et al., 2015). However, some common STS regulators interact with more than their primary pathway. Aside from regulating nitrogen assimilation, NtrB/NtrC regulates flagellar activity (Taylor et al., 2015), and EnvZ/OmpR, which directs osmoregulation, also regulates pathogenesis in *Escherichia coli* with *Drosophila melanogaster* (Pukklay et al., 2013). In addition to regulating different phenotypes through divergent signaling pathways, HKs can converge onto similar pathways. For example, hybrid HKs can utilize non-cognate HPt domains to elicit responses (Chambonnier et al., 2016; Norsworthy & Visick, 2015). This multi-functionality is possible due to their structural integrity and conserved domain wiring. The general flow of phosphorylation in cross-talking proteins is depicted in Figure 1C.

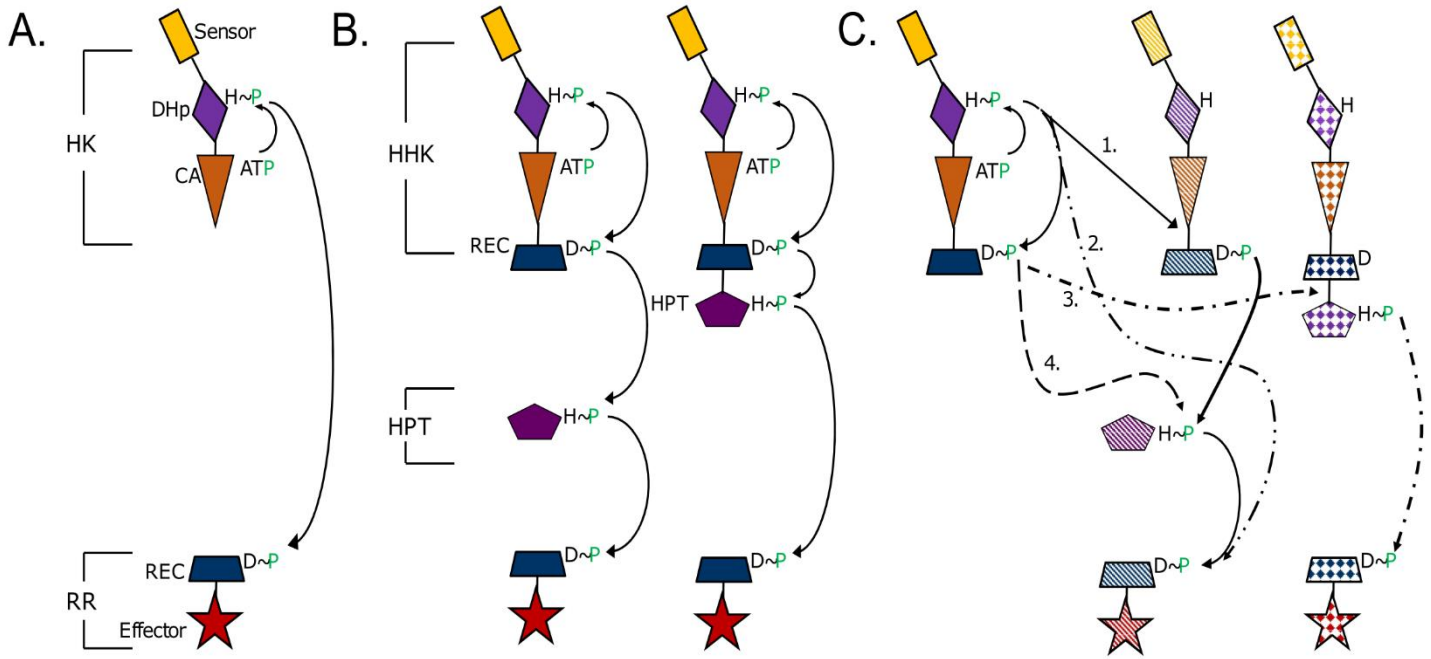


Figure 1. Diagrams depicting the prototypical flow of phosphates moieties from histidine kinases (HK) and hybrid histidine kinases (HHK).

Yellow rectangle: Sensory domain; Purple diamond: Dimerization and histidine containing domain; Orange triangle: catalytic HATPase domain; Blue rhombus: Receiver and aspartate containing domain; Red star: Effector domain; Purple pentagon: Histidine phosphotransferase domain or protein. **A)** Classic view of HKs and their phosphorylation to a downstream response regulator (RR) protein. **B)** Classic view of HHKs and their phosphorylation cascade via an intermediate histidine phosphotransferase (HPT) protein or C-terminal HPT domain to the downstream RR. **C)** Cross talk between HHKs to a different pathway via 1. A different REC domain on a second HHK (solid line); 2. Bypassing a HHK REC domain and phosphorylating the RR from the HHK Dhp domain (dash two dotted line); 3. Using the given REC domain to phosphorylate another HHK's HPT domain – RR pair (dash one dotted line). 4. Using the given REC domain to phosphorylate a different HPT – RR pair (dashed line). Note: HKs, HHKs, and RRs are homodimeric proteins but here are illustrated as only one part of the homodimer. See text for specific details on mechanism.

I.b. Domain function in signal transduction phosphorylation

STS sensory domains are complex, come in varying styles and arrangements, and act as receptors for ligands ranging from small molecules to environmental cues (Cheung & Hendrickson, 2010). Two of the more common sensory domains are the Cache and PAS domains. Although less studied, the Cache domain binds small molecules and is named for the Ca^{2+} channel and chemotaxis proteins in which it was first identified (Anantharaman & Aravind, 2000). The PAS domain (Per-Arnt-Sim) localizes to either the periplasm or cytoplasm (Etzkorn et al., 2008) in proteins sometimes responsible for redox reactions and light response (Taylor & Zhulin, 1999). Though the modular architecture depends on the HK, the Cache and PAS domains are commonly associated with methyl-accepting chemotaxis proteins (Anantharaman & Aravind, 2000; Mascher et al., 2006) or with the LuxQ/P family of proteins, where in some instances the sensory domain binds periplasmic proteins that act as signal receptors (Neiditch et al., 2005).

Little is known overall about signal perception among HKs, but insights into their general sensory architecture gives rise to some mechanistic understanding. STSs are typically anchored within the transmembrane region where a "linker region" connects the periplasmic sensory domain to the rest of the protein in the cytoplasm (Mascher et al., 2006), transmitting the accepted signal. The HAMP domain is one common linker, named after the proteins where it is found: HKs, Adenylyl cyclases, Methyl-carrier proteins, and Phosphatases (Aravind & Ponting, 1999). HAMP domains are found in singular or tandem arrays, typically in conserved clusters as a continuation of transmembrane helices (Dunin-Horkawicz & Lupas, 2010) and usually follow after PAS or Cache domains (Szurmant et al., 2007). The HAMP domain converts signal to HK activity by acting as a gear shift, rotating via the common knobs-into-holes and complementary

x-da packing (Hulko et al., 2006) or a unique ridges-into-grooves interaction, transitioning from a compact to less compact state (Airola et al., 2010). The HAMP domain positions the downstream domains into functional arrangements through intermolecular recognition to regulate kinase activity (Ferris et al., 2012; Stewart, 2014).

After the HAMP-induced conformational change, the catalytic domains, which are categorized by two main domains, the HisKA (also called the Dhp domain) and HATPase domain, are activated (Zschiedrich et al., 2016). The HATPase domain is positioned to cleave the γ-phosphate from ATP where it is transferred to the conserved His in the HisKA domain. The position of the His residue depends on the spatial arrangement of the particular HK, where only one or both of the two His residues in the homodimer are exposed in the cytoplasm (Tomomori et al., 1999). These two positional arrangements are referred to as *cis* or *trans* phosphorylation (Ashenberg et al., 2013; Zschiedrich et al., 2016), where *trans* phosphorylation appears more favorable (Marina et al., 2005). Once the HATPase-HisKA complex relaxes after phosphorylation, the RR enters the complex and catalyzes the transfer of the phosphate to its own highly conserved Asp in the REC domain. The RR then typically acts as a transcriptional switch, regulating a myriad of cell physiological responses (Bourret, 2010) through C-terminal effector domains such as DNA binding domains and enzymatic domains (Galperin, 2010).

Though the prototypical mechanisms and general effects are well established for many STS proteins, little is known about what drives their phosphorylation and regulation. Phosphorylation appears more dependent on the fluctuation of the phosphoryl groups rather than the stoichiometry of the proteins, as only a small number of HKs in the cell are phosphorylated at a given time, in part due to the high energy in the N~P bond in the conserved His residue (Stock et al., 2000). Similarly, the high-energy acyl-phosphates on the conserved Asp residue of RR

proteins more rapidly hydrolyze compared to the His~P. This rapid hydrolysis is thought to drive the conformational changes within the protein structure, where stabilizing the bond can extend the half-life of the Asp~P in the RR (Stock et al., 2000).

The RR-REC domain catalyzes autophosphorylation from HKs or small phosphor-donors**.** Though small phospho-donors do not provide much physiological relevance, they could provide a link to metabolism and other STS regulation (Bourret, 2010). The interaction and regulation of STS proteins through phosphorylation appear to be an intricate web of control, whether at the stage of phosphates or the activity of the proteins. Further work will add to the understanding of how this diverse protein family establishes its hold on cellular regulation through environmental cues.

I.c. Evolution and cross-talk in STS pathways

The evolutionary histories of STS proteins originated in bacteria and have since radiated to the other Kingdoms through lateral events (Wuichet et al., 2010). In prokaryotic genomes, HKs and RRs are generally located near each other and scale in number with the size of the genome, suggesting coevolution. However, this is only a general rule, since some systems will utilize available orphan HKs and/or other regulators to elicit responses (Petters et al., 2012; Pankey, Foxall et al., 2017; Steiner et al., 2011).

One major unknown of these highly conserved STS HisKA and REC domains is the process by which they maintain sufficient signaling isolation or partner specificity to avoid deleterious outcomes from cross-talk between pathways. This question has driven research toward the evolutionary history of these proteins. Although widely varied and typically isolated

in their specific functions, these genes generally evolve from horizontal gene transfer (HGT) and gene duplication, and their protein domain architecture through domain shuffling. These three phenomena - along with subfunctionalization and diversification (Proulx, 2012) - drive the isolation and rise of paralogous STS in signaling pathways (Alm et al., 2006; Koretke et al., 2000; Salazar & Laub, 2015; Zhang & Shi, 2005).

Although the coevolution of single HK-RR STSs appears straightforward**,** the evolutionary history of hybrid HKs remains undetermined. At first, all hybrid subfamilies belonged to one clade (Koretke et al., 2000), but phylogenetically unrelated members within the "hybrid" clade arose where the kinase and receiver domains did not belong to the same subfamily (Zhang & Shi, 2005). This suggested that these family members obtained domains through lateral transfer events and not from a common ancestor. Domains of some hybrid HKs displayed high sequence similarity suggesting the genes were duplicated before undergoing subfunctionalization**.** While hybrids underwent lateral domain movement and acquisition, phylogenetic analysis of HPt proteins reveal they share a common ancestor, most likely due to their conserved domain structure and active site motif (Zhang & Shi, 2005). It is remarkable that these systems, despite sharing different evolutionary histories, work in tandem to elicit important cellular responses.

Bacteria that experience rapid environmental changes typically have greater STS content within their genome (Capra & Laub, 2012) and utilize multiple systems to sense the plethora of environmental stimuli. One way to detect a new signal is through domain shuffling that occurs during or prior to a gene duplication, where most paralogs show low conservation between sensory domains (Capra & Laub, 2012). In conjunction with evolving sensory domains, output domains can potentially recognize new targets or different sequences within the genome. This is

critical for evolution, as RRs are responsible for directly controlling physiological responses. For example, evolution can occur in the DNA-binding site leading to different transcriptional effects in different species (Capra & Laub, 2012).

Aside from the convoluted evolutionary history of the domains, the phosphotransfer mechanism also experiences selective pressures that drive evolution. Protein phosphotransfer ability is strongly linked to the preferred interacting partner, suggesting the ability to signal depends on the molecular recognition of the partners. However, residue coevolution is a rare event since the molecular interface between the HK-RR pair can tolerate some mutations to preserve phosphorylation transfer since deleterious mutations could hinder the interaction (Capra et al., 2010). Thus, the HK-RR must undergo neutral evolution to ensure isolation after gene duplication to preserve cellular function. With hybrid HKs, the extra REC domain adds a new dimension to the spatial arrangement of the protein, thus possibly creating a different selection force on the coevolving residues, but at the same time re-enforcing the specific interaction; without the REC domain, the hybrid HK could be more promiscuous (Capra & Laub, 2012).

I.d. *Euprymna scolopes* – *Vibrio fischeri* symbiosis model

The partnership between the bacterial species *Vibrio fischeri* and its eukaryotic host *Euprymna scolopes*, the Hawaiian bobtail squid, has been a key model for studying the molecular intricacies of bacteria-eukaryote symbiosis (McFall-Ngai & Ruby, 1991). *V. fischeri*, a bioluminescent marine bacterium, colonizes the squid's light organ to provide counter-illumination against the down-welling moonlight, providing camouflage while the squid hunts (Jones & Nishiguchi, 2004). Reciprocally, the squid provides nutrients and branched-chain

amino acids for the bacteria, supporting a dense population and bioluminescence (Graf & Ruby, 1998). This system has proven highly effective for the study of both how genes and molecular pathways affect squid colonization and how pathways that affect symbiosis and new niches are established (Geszvain et al., 2005; Visick & Ruby, 2006).

The symbiosis is established in three phases: initiation, colonization, and persistence (Nyholm & McFall-Ngai, 2004). During initiation, the squid horizontally acquires the bacterial symbionts which can form an aggregate in squid-derived mucus that collects outside the light organ pores, although only a few cells initiate colonization (Altura et al., 2013; Nyholm et al., 2000; Wollenberg & Ruby, 2009). In the aggregate these bacteria are motile and use flagella to navigate from the pores through the ducts into the light organ crypts (Graf et al., 1994; Millikan & Ruby, 2002). Navigating through the ducts, these bacteria battle host defenses including oxidative stress in the form of peroxides and nitric oxide (Davidson et al., 2004; Tomarev et al., 1993) and recognition, attachment and engulfment by host hemocytes (McFall-Ngai et al., 2010; Nyholm et al., 2009). *V. fischeri* transitions into the colonization phase in the light organ crypts where they produce a dim light through bioluminescence, the cornerstone of the partnership (McFall-Ngai et al., 2012). Reaching the crypts is not enough, as *V. fischeri* must withstand and persist after a daily venting where 95% of the bacteria population are ejected and the 5% remaining must regrow to maintain a population (Nyholm & McFall-Ngai, 2004).

STSs not only regulate bioluminescence in the symbiosis, but also biofilm formation, an important symbiotic phenotype. Biofilm matrices are essential for aggregation in the initiation phase and mainly formed by two carbohydrates: a symbiotic polysaccharide (Syp) and cellulose (Shibata et al., 2012; Yip et al., 2005). Syp biofilm is encoded by a multi-operon locus controlled by the hybrid HK regulator RscS (Geszvain & Visick, 2008; Visick & Skoufos, 2001; Yip et al.,

2006). This regulator cross-signals to a second hybrid HK, SypF (Norsworthy & Visick, 2015), which phosphorylates SypE, SypG (Hussa et al., 2008), and VpsR RRs (Darnell et al., 2008). Once phosphorylated, SypG activates four promoters across the Syp locus, turning on genes important for Syp structure, regulation, and export (Ray et al., 2013). SypE exhibits positive and negative regulatory effects on biofilm formation (Morris et al., 2011) and currently signals to the non-STS regulator SypA. Though little is known about SypA function, the result is repressed biofilm formation (Morris & Visick, 2013a, 2013b). The two-component regulation of Syp biofilm is highly intricate, as seen through the recent work connecting the quorum sensing regulon to Syp through LuxU and SypK (Miyashiro et al., 2014; Ray & Visick, 2012). Some *V. fischeri* strains, such as MJ11 (Haygood et al., 1984), do not encode RscS in their genome; therefore pre-existing conditions where bacterial strains are genetically pre-wired to bridge the gap of missing symbiotic regulators may exist (Pankey, Foxall et al., 2017).

V. fischeri niches range from planktonic / free living to eukaryotic hosts, such as the squid and fish light organ. The strains from these different niches vary in levels of symbiotic colonization capacity with the Hawaiian bobtail squid, where some strains are naïve at navigating host defenses compared to the native squid strain ES114 (Ruby & Lee, 1998; McFall-Ngai & Ruby, 1991). This natural variation was utilized to study the potential of evolutionary pre-wiring to influence the niche expansion ability of squid-naïve strains for symbiosis with *E. scolopes*, thereby revealing unknown mechanisms of host-symbiont partner selection and specificity (Pankey, Foxall et al., 2017). The strains used in this study include MJ11, isolated from the light organ of *Monocentris japonica*; H905, a planktonic isolate located in the same waters as ES114; WH1, a Massachusetts plankton isolate; EM17, a *Euprymna morseii* light organ isolate; and the native symbiotic strain ES114 (Pankey, Foxall et al., 2017).

This evolution approach uses the squid's innate ability to horizontally select a symbiont capable of surviving the rigorous requirements for colonization: aggregation in a biofilm, oxidative stress resistance, evasion of host hemocytes, and persistence within the light organ through daily venting (Fig. 2). Briefly, bacteria were inoculated into a communal bowl with newly hatched squid juveniles and incubated overnight. After the first squid light organ venting, serving as the first bottlenecking event, the squid were separated into different lineages allowing each squid light organ to serve as an isolated parallel evolving population. Each squid would subject its population to subsequent cycles of venting and remaining bacteria would repopulate the light organ. Following four days and four light organ venting cycles, the bacterial population expelled from this first light organ were introduced to new hatching squid and serially passaged through a total of 15 squid. This experiment was estimated to contain 60 bottlenecking events and 290-360 bacterial generations (Pankey, Foxall et al., 2017; Schuster et al., 2010). Representative bacteria were isolated from each squid light organ lineage at passage 15 and at earlier passages. These isolates were characterized for phenotypes convergent with the native symbiont ES114 and for squid improved colonization as compared to its ancestor and ES114. The experiment revealed that the strains with the greatest starting symbiotic deficit rapidly evolved and dramatically improved in symbiotic potential, and attained traits that were convergent with the native strain (Pankey, Foxall et al., 2017; Schuster et al., 2010).

Subsequent genomic resequencing identified single point mutations across all populations within MJ11 and more dramatic changes including gene deletions in H905 converged to a single locus. In contrast very few mutations occurred in EM17, WH1, or ES114 (Pankey, Foxall et al., 2017). The locus wherein convergent mutations arose was identified as *binK*, and was first identified as a negative regulator of biofilm in H905 (Perry, 2009) and MJ11 (Ster, 2015) then

subsequently identified in the native strain ES114 (Brooks & Mandel, 2016). The evolution approach further revealed that *binK*, through its influence on both syp and cellulose, represses aggregation outside the light organ pores, impairs protection against oxidative stress and host hemocyte attachment, and lowers the quorum threshold to enhance luminescence (Pankey, Foxall et al., 2017). Although these mechanisms and functions have not been fully characterized, STS pathways in bacterial and eukaryotic symbiosis are clearly essential, and *binK*, being a global regulator of these traits, allowed for an exceptional leap to symbiosis of these squid naïve strains.

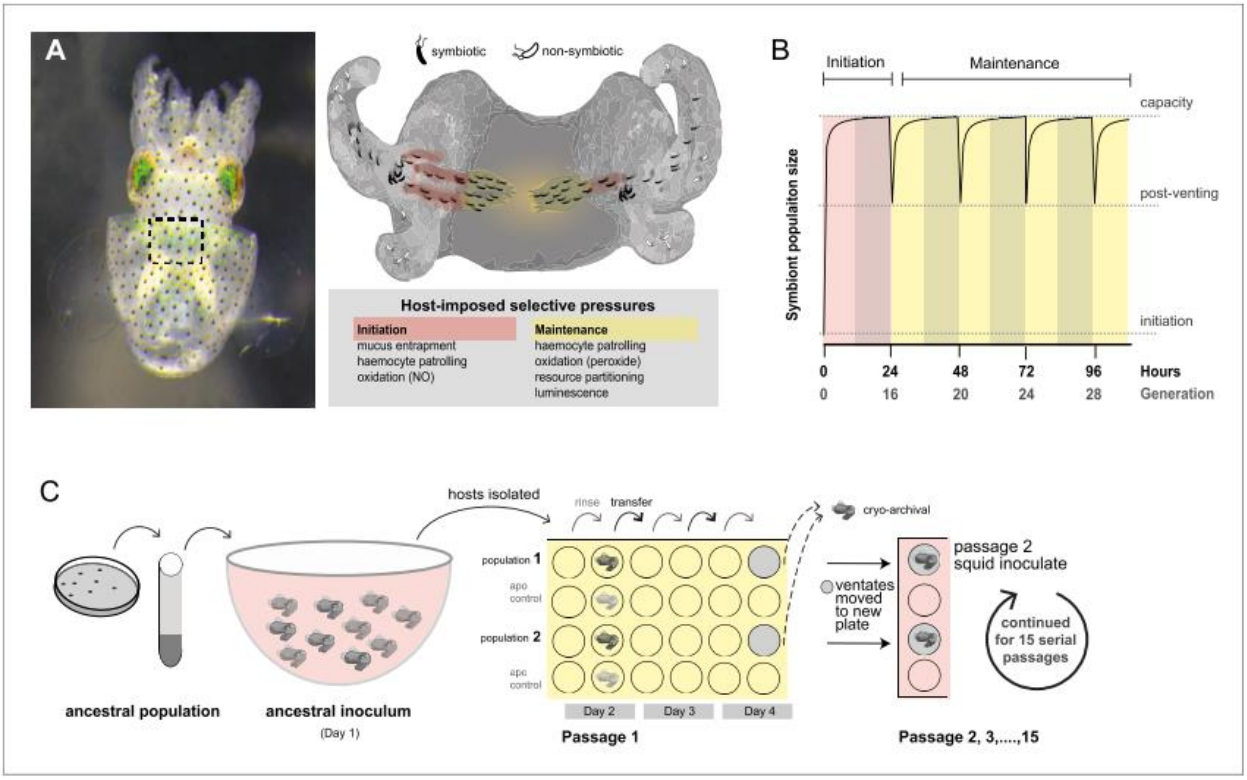


Figure 2. Host selection mechanisms that shape adaptive evolution by *V. fischeri*.

A) Dorsal view of juvenile host *E. scolopes* (left) with box indicating the relative position of the ventrally situated symbiotic light organ. On the right, a schematic illustrating the stages at which host-imposed selection occurs during squid–*V. fischeri* symbiosis: host recruitment (mucus entrapment, aggregation at light organ pores), initiation of symbiosis (host defenses, including hemocyte engulfment and oxidative stress), and colonization and maintenance (nutrient provisioning, sanctioning of non-luminous cheaters, continued hemocyte patrolling, and daily purging). **B)** Symbiont population growth modeled for a single passage on the basis of growth dynamics of *V. fischeri* ES114. Light-organ populations are initiated with as few as ~10 cells (Wollenberg and Ruby, 2009; Altura et al., 2013) or as much as 1% of the inoculum, but are reduced by 95% following venting of the light organ at dawn (every 24 hr) (Boettcher et al., 1996). Shaded areas represent night periods whereas light areas represent daylight, which induces the venting behavior. **C)** Experimental evolution of *V. fischeri* under host selection as described in Schuster et al. (2010). Each ancestral *V. fischeri* population was prepared by recovering cells from five colonies, growing them to mid-log phase, and sub-culturing them into 100 mL filtered seawater at a concentration sufficient to colonize squid (≤20,000 CFU/mL). On day 1, ten un- colonized (non-luminous) juvenile squid were communally inoculated by overnight incubation, during which bacteria were subjected to the first host- selective bottleneck. Following venting of ~95% of the light organ population, the squid were separated into isolated lineages in individual wells of a 24-well polystyrene plate containing filtered sea water with intervening rows of squid from an un-inoculated control cohort, the aposymbiotc control (‘apo control’). Note that only two of the ten passage squid populations are shown. On days 2, 3, and 4, after venting, squid were rinsed and transferred into 2 mL fresh filtered seawater. Luminescence was measured at various intervals for each squid to monitor colonization and the absence of contamination in aposymbiotic control squid. On the fourth day, the squid and half of the ventate were frozen at -80˚C to preserve bacteria, and the remaining 1 mL ventate was combined with 1 mL of fresh filtered seawater, and used to inoculate a new uncolonized 24-hr-old juvenile squid. The process continued for 15 squid only for those lineages in which squid were detectably luminous at 48 hr post inoculation. This figure is found in Pankey, Foxall et al. (2017).

I.e. Quorum sensing in bioluminescence

The cornerstone of the squid – *Vibrio* symbiosis is the ability of *V. fischeri* to bioluminesce. Bioluminescence occurs through quorum sensing, a cell population based mechanism where bacteria sense a density cue to elicit a cellular response. As first documented in *V. fischeri* and is sometimes referred to as pheromone sensing, quorum sensing occurs through the recognition of acyl-homoserine lactone (acyl-HSL) autoinducer (AI) molecules (Eberhard et al., 1981). This earned *V. fischeri* a role as the key organism in understanding bacterial quorum sensing signaling mechanisms. *V. fischeri* and *V. harveyi'*s homologous quorum sensing systems (Table 1), built through extensive genetic and biochemical functional studies, formed the current understanding of quorum sensing through the central *luxUO* regulatory node that integrates signal transduction from multiple sensory systems.

As first hypothesized by Lupp et al. (2003), and upheld as the current working model, quorum sensing in *V. fischeri* is a three-stage cell density sensing process induced by the "*ain* and *lux*" system (Lupp & Ruby, 2004; Lupp et al., 2003; Fig. 3). First, at low cell density (Fig. 3A), the AI molecules *N*-3-oxo-hexanoyl homoserine lactone (3OC6-HSL), *N*-octanoyl-homoserine lactone (C8-HSL), and furanosyl borate diester (AI-2) are in low abundance. Consequently, the core STS of AinR, LuxQ, LuxU, and LuxO, represses luminescence. The DNA-binding domain of the RR LuxO (Freeman & Bassler, 1999; Miller & Bassler, 2001; Miyamoto et al., 2003; Miyamoto et al., 2000), when phosphorylated by the upstream hybrid HKs AinR (homologous to *V. harveyi*'s LuxN; Freeman et al., 2000) and LuxQ via the HPt LuxU, up-regulates transcription of a small RNA *qrr1* (Miyashiro et al., 2010). High abundance

of *qrr1* blocks transcription of *litR*, the homolog to *V. harveyi*'s LuxR, which then up-regulates the *lux* operon via up-regulating LuxR (not homologous to *V. harveyi*'s LuxR; Table 1).

As the population builds to a moderate cell density (Fig. 3B), the AI synthases AinS and LuxS produce more C8-HSL (Gilson et al., 1995; Kuo et al., 1994; Kuo et al., 1996) and AI-2 (Chen et al., 2002; Miller & Bassler, 2001), respectively. Once C8-HSL and AI-2 reach the activity threshold, they bind to their respective receptors AinR (Kimbrough & Stabb, 2013; Lupp et al., 2003) and LuxQ/P (Miyashiro et al., 2014; Neiditch et al., 2005). This binding triggers the AinR and LuxQ phosphatase activity to take over by significantly lowering their kinase activity (Freeman et al., 2000; Timmen et al., 2006). The phosphatase activity by AinR and LuxQ, unaffected by AI activity, dephosphorylates LuxU and subsequently leaves LuxO un-phosphorylated and deactivated (Timmen et al., 2006). LuxO deactivation increases *litR* transcription through down-regulation *qrr1* expression. LitR then activates the expression of LuxR (Fidopiastis et al., 2002), which first accepts the C8-HSL signals produced by AinS. After homodimerization, LuxR directly binds to the "lux box" promoter region and up-regulates the *lux* operon, resulting in luminescence induction (Antunes et al., 2008; Fuqua et al., 2001; Miller & Bassler, 2001; Miyashiro & Ruby, 2012).

Finally, at a high cell population density (Fig. 3C), the *lux* operon is fully induced, including *luxI* transcription. Increased LuxI produces more 3OC6-HSL, which outcompetes C8-HSL for LuxR reception and effectively ramps up *lux* transcription to fully induce luminescence (Colton et al., 2015; Miyashiro & Ruby, 2012).

Although these three systems (AinS/R, LuxS/Q, LuxI/R) are the key regulons of quorum sensing in *V. fischeri* by AIs, luminescence is also regulated by environmental cues, such as the redox-responsive system ArcA/B (Bose et al., 2007). In this system, ArcB phosphorylates ArcA,

directly blocking *lux* transcription and therefore repressing luminescence (Fig. 3D). During colonization of *V. fischeri* in the squid *E. scolopes,* the ArcA/B system responds to the oxidative stress produced by the squid's light organ, de-repressing *lux* transcription and allowing AIs to induce luminescence. At later stages of colonization and when the cells are at high density**,** as the oxidative species lessen, ArcA/B is turned on but cannot outcompete the activated LuxR for the *lux* box, thus leaving luminescence fully induced (Fig. 3E) (Bose et al., 2007). The ArcA/B system plays into the positive feedback loop of luminescence induction by 3OC6-HSLs whereby once luminescence is fully induced ArcA cannot overcome it, similar to the squid's light organ (Septer et al., 2012). However, the ArcA/B repression of luminescence is relatively weak and in the symbiosis of the squid by *V. fischeri* only a sub population need to be producing 3OC6-HSL to fully induce luminescence (Septer et al., 2012).

The global regulator RR GacA (Whistler & Ruby, 2003) also represses luminescence in *V. fischeri*. GacA facilitates repression through CsrA which activates two RNAs *csrB1* and *csrB2* that block CsrA's ability to bind to the *luxI* transcript, thereby blocking production of the 3OC6-HSL signal and reducing transcription and translation (Fig. 3F) (Ballok, 2007). Though GacA is part of the GacS/A STS that is well established in *E. coli*, GacA is not mediated by GacS to actively represses luminescence because a GacS mutant does not have the same phenotypes as a GacA mutant (Septer et al., 2015). GacS is suspected to independently affect luminescence by an unknown mechanism, separate from GacA, and presumably through accumulation of citrate (Septer et al., 2015).

Although quorum sensing is well studied in the native symbiont strain ES114, there are fundamental differences in luminescence regulation in other strains. The main examples currently studied are ES114, MJ1, and MJ11, the latter two being isolates from *M. japonica*.

Through empirical modeling of *luxR* allelic variants found in MJ1 and ES114, Colton et al. (2015) found that divergent evolution drove LuxR to respond differently to the presence of 3OC6- and C8-HSL. MJ11, when compared to ES114, produces more 3OC6-HSL and less C8-HSL (Miyashiro & Ruby, 2012). In addition to the divergent evolution between *luxR* in these two strains, MJ11's intergenic region between *luxR* and *luxI* is under divergent and rapid evolution, explaining some strain brightness variations among the *V. fischeri* species (Bose et al., 2011).

With this in-depth understanding of quorum sensing, more is revealed on how other proteins interact with the dominant circuitry, and how these other proteins integrate other signals important for the regulatory decision over cell density. In the pathogenic quorum sensing systems in *V. harveyi* and *V. cholera*, the LuxU/O system regulates luminescence and biofilm formation, as well as pathogenicity factors (Hammer & Bassler, 2003; Lilley & Bassler, 2000; Miller et al., 2002; Mok et al., 2003; Zhu et al., 2001). Within the last decade, two hybrid HKs, CqsR and VpsS, were discovered to also interact with the LuxU node in the circuity of *V. cholera* (Jung et al., 2015; Shikuma et al., 2009). This finding sets a precedent for the same to be possible in *V. fischeri*, where LuxU is activated by more hybrid HKs. Although these other kinases in *V. cholera* activated LuxU at a lower efficiency, these other sensors further paint quorum sensing as an intricate link between multiple cellular processes and regulators.

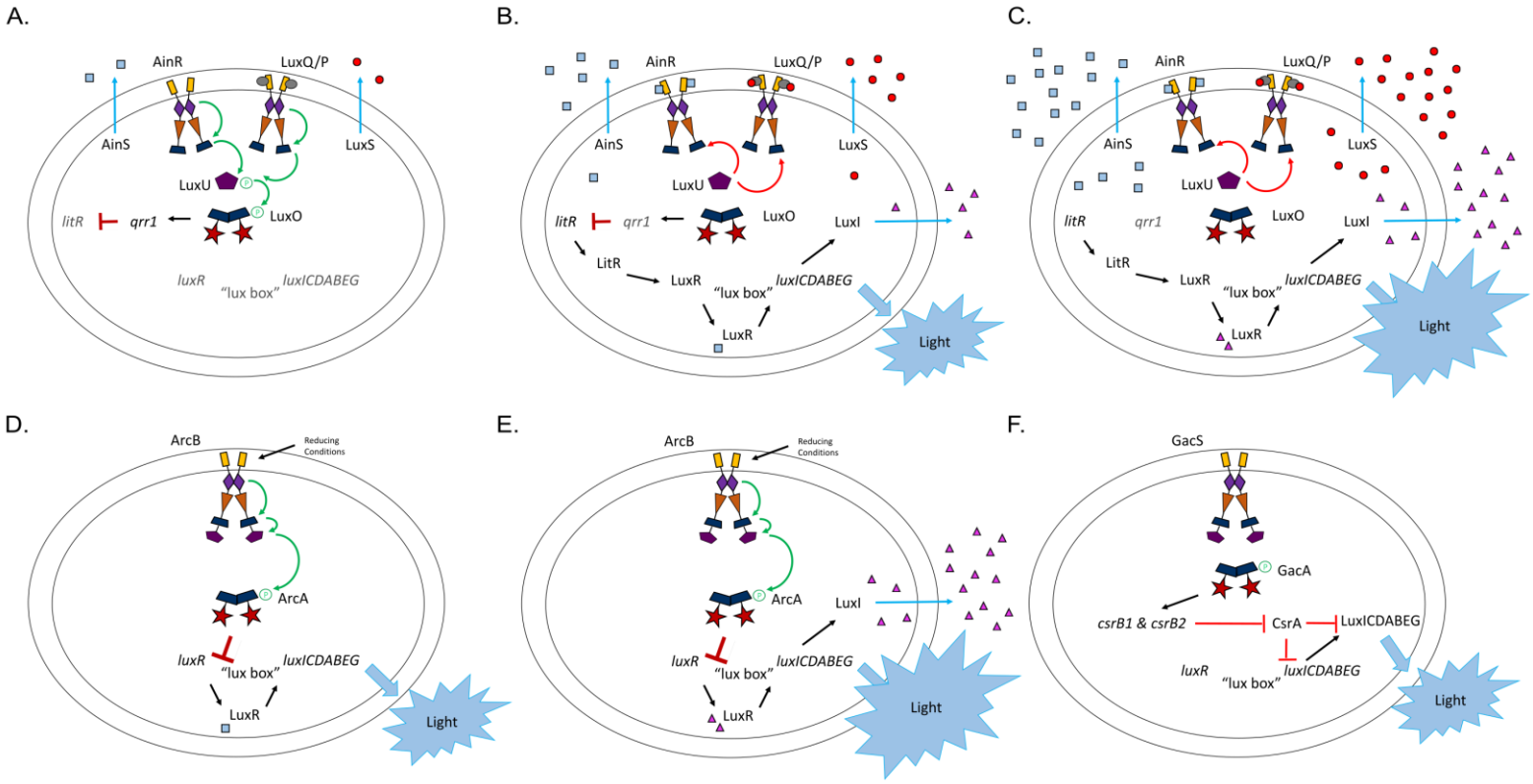


Figure 3. Two-Component signaling control of bioluminescence in *V. fischeri.*

A-C) Quorum sensing control of luminescence. **A)** At low cell density, the autoinducer molecules are in low abundance (C8-HSL – blue square; AI2 – red circle; 3OC6-HSL – pink triangle), and HHKs AinR and LuxQ act as kinases, phosphorylating (green arrow) the HPT LuxU and subsequently the RR LuxO, upregulating expression of *qrr1*, and blocking transcription of *litR*, repressing luminescence. **B)** At moderate cell density, the autoinducer signals increase in abundance and bind to their respective regulator (C8-HSL to AinR and AI2 to LuxQ/P). Upon sensing signals, the kinase activity switches to phosphatase activity, dephosphorylating LuxU and inducing luminescence by upregulating *luxR* by LitR, where LuxR responds to C8-HSL. **C)** At high cell density, the autoinducers reach a threshold and freely move in and out of the cell, 3OC6-HSL outcompetes C8-HSL for LuxR binding and fully induces luminescence. **D-E)** Weak luminescence repression as seen in *V. fischeri* symbiont strain ES114 during reducing conditions by the HHK ArcB - RR ArcA where ArcA cannot override luminescence induction in either presence of **D)** C8 which is indicative of culture induction where luminescence is dimmer, or **E)** in the squid light organ where 3OC6 fully induces luminescence. **F)** Luminescence repression by the RR GacA through small RNAs *csrB1* and *csrB2* sequestering CsrA from blocking transcription and translation of the lux genes required for luminescence. The colored shapes that depict the protein domains are described in Figure 1.

Table 1. Homologous quorum sensing proteins between *Vibrio fischeri* and *V. harveyi.*

V. fischeri	**Function**	***V. harveyi***	**Function**
AinR	Receptor for C8-HSL; Regulates LuxU phosphorylation	LuxN	Receptor for AI-1; Regulates LuxU phosphorylation
LuxQ	Regulates LuxU phosphorylation	LuxQ	Regulates LuxU phosphorylation
LuxP	Receptor for AI-2	LuxP	Receptor for AI-2
LuxU	Regulates LuxO phosphorylation	LuxU	Regulates LuxO phosphorylation
LuxO	Regulates *qrr1* transcription	LuxO	Regulates *qrr1-4* transcription
LitR	Regulates LuxR transcription	LuxR	Directly regulates luminescence
LuxR	Directly regulates luminescence	-	-

Abbreviations: C8-HSL (*N*-octanoyl homoserine lactone); 3OC6-HSL (N-3-oxo-hexanoyl homoserine lactone); AI-1 (*N*-(D-3-hydroxybutanoyl) homoserine lactone); AI-2 (furanosyl borate diester)

Specific Aims

1. Identify downstream interacting partners and mediators of BinK regulation
2. Define how BinK interfaces with the quorum sensing regulatory cascade to influence luminescence

I.f. Specific Aim 1: Identify downstream interacting partners and mediators of BinK regulation

The first specific aim is to identify the interacting partners of BinK. As shown through our work and the work of colleagues, BinK exerts control over two primary traits: repression of biofilm formation and activation of bioluminescence through altered quorum sensing autoinducer levels (Pankey, Foxall et al., 2017). Loss of *binK* (*ΔbinK* mutant) decreases luminescence and 3OC6-HSL levels. It is unknown what protein(s) interact with BinK to elicit this luminescence effect. This work will investigate how BinK functions in the bioluminescence pathway and illuminate whether it partners with another STS protein in the quorum sensing regulon, as in *V. cholera* (Jung et al., 2015; Shikuma et al., 2009).

To identify how BinK affects quorum sensing regulation of bioluminescence, *binK* will be over-expressed in null RR and quorum sensing mutants. In a wild-type ES114, BinK activates luminescence; if a potential downstream RR partner is missing, it is expected BinK will not increase luminescence. In addition to standard luminescence assays, the expression of the small RNA *qrr1* will also be studied. To test if the altered AI levels that were previously established (Pankey, Foxall et al., 2017) is a result from upstream regulatory effects, such as *qrr1* regulation, plasmids encoding a *qrr1* promoter fusion to a reported gene *gfp* will be used to measure *qrr1*

expression in various *V. fischeri* strain backgrounds. We hypothesize that *qrr1* levels will be higher in a Δ*binK* background at lower cell densities when compared to wild-type *qrr1* levels.

I.g. Specific Aim 2: Define how BinK interfaces with the quorum sensing regulatory cascade to influence luminescence

This second aim is to test functionality of BinK as a STS hybrid HK. BinK contains a C-terminal REC domain, so to test function of BinK as a hybrid HK, we will examine BinK in the absence of the REC domain and examine the REC domain alone. Though hybrid HKs are generally hypothesized to use their C-terminal REC domain for forward and reverse phosphate flow, the RscS activator of biofilm and Syp induction does not; instead, it cross-signals to the HPt domain of SypF (pathway 3 in Fig. 1C; Norsworthy & Visick, 2015). Although RscS does use its REC domain as typically expected from hybrid HKs, there is precedent for BinK to not require its C-terminal end for functionality. Thus, the goal is to characterize said function and to investigate whether BinK acts as a canonical hybrid HK that requires the REC domain to function.

BinK function will be tested in terms of its role in bioluminescence activation. In order to test REC domain functionality, variant alleles will be generated. A truncated BinK allele ($BinK^{\Delta REC}$) will convert BinK to a typical HK, while the BinK REC domain fused to the *binK* promoter (P_{binK}:REC) will test the activity of just the REC domain in luminescence activation. The absence of the REC domain is hypothesized to lead to no activation of luminescence while the REC domain alone will increase luminescence, similar to LuxN activity (Freeman et al., 2000). In addition to REC variants, point mutant alleles will be generated to further investigate activity. The histidine to glutamine (H362Q) and aspartate to alanine (D794A) mutants will

respectively affect kinase and phosphatase ability, and a third double point mutant (H362Q/D794A) will act as a null allele with no functional ability. We hypothesize that H362Q will abolish kinase activity but leave phosphatase activity intact, thus activating luminescence. The D794A point mutant will abolish phosphatase activity and also will not activate luminescence, and the double point mutant will generate a null function allele and lead to no activation of luminescence.

II. METHODS

II.a. Bacterial strains and growth conditions

The bacterial strains (*Vibrio fischeri* and *Escherichia coli*) and plasmids used in this study are listed in Tables 2, 3, and 4, respectively. All *V. fischeri* strains were routinely grown in LBS (1% tryptone, 0.5% yeast extract, 2% NaCl, per 1L diH_2O) with shaking or on 1.5% agar plates at 28°C overnight. Experiment cultures were grown in seawater-tryptone (SWT) medium (0.5% tryptone, 0.3% yeast extract, 0.3% glycerol, 70% instant ocean (IO) at 32ppt, per 1L diH_2O) for MJ11 strains, or SWTO (SWT with 1% NaCl and 78% IO at 32ppt, per 1L diH_2O, Bose et al., 2007) for ES114 strains. *V. fischeri* was also grown in HEPES minimal medium (HMM; Ruby & Nealson, 1976), a seawater-based minimal medium with 1x artificial sea water (ASW: 50mM $MgSO_4$, 10mM CaCl2, 300mM NaCl, 10mM KCl), 0.333mM K_2HPO_4, 18.5mM NH_4Cl, 0.0144% Casamino acids, 32.6mM glycerol, and 10µM ferrous iron solution buffered with 10mM HEPES. *E. coli* cells were used to maintain plasmids and were grown on LB (1% tryptone, 0.5% yeast extract, 1% NaCl, per 1L diH_2O) either on 1.5% agar or liquid, or in brain heart infusion (BHI) medium. The following antibiotics were used for plasmid selection when needed: Chloramphenicol (Cm, 2.5µg/mL for *V. fischeri* and 25µg/mL for *E. coli*), Erythromycin (Erm, 5µg/mL for *V. fischeri* and 150µg/mL for *E. coli* in BHI), Kanamycin (Kan, 50µg/mL for *V. fischeri* and *E. coli*). Plasmids were moved from *E. coli* cells into *V. fischeri* strains via tri-parental conjugation as previously described (Stabb & Ruby, 2002).

II.b. Molecular DNA technologies and PCR

DNA amplifications by PCR and site directed mutagenesis utilized oligonucleotide primers synthesized by Integrated DNA Technologies (Coralville, IA; Table 5). PCR amplification was conducted using a Master Cycler Nexus thermocycler (Eppendorf, Hamburg, Germany) per manufacturer protocols. Phusion High Fidelity DNA Polymerase (Finzyme for pIMS1A4; Thermo Scientific for all others) was used for generating PCR amplicons that were subsequently cloned or for Splicing and Overlap Extension (SOE) PCR (Horton et al., 1990) whereas routine PCR screening was performed with AccuStartII Supermix (Quanta BioSciences, Gaithersburg, MD). Point mutant variants were generated with QuikChange II XL site directed mutagenesis kit (Agilent Technologies). When applicable, PCR fragments were purified with a QIAquick kit (Qiagen, Valencia, CA) and SOE products were fused using Expand Long Template DNA polymerase (Roche) after initial amplification with a Phusion High Fidelity polymerase. PCR fragments amplified through Phusion or Expand Long Template, unless otherwise stated, were cloned into pCR™2.1TOPO®, then transformed into *E. coli*. All TOPO constructs were sequenced for 1x coverage at GeneWiz (South Plainfield, NJ) unless otherwise stated. Annealing temperatures used for PCR amplification were determined by subtracting 1°C from the lowest melting temperature in the primer pair, as calculated by Premiere Biosoft's Netprimer (http://www.premierbiosoft.com/netprimer/index.html).

Standard molecular methods of genomic and plasmid DNA isolation, transformation, restriction enzyme digests, gel electrophoresis, and ligation followed protocols supplied by manufacturers or previously published. Transformations used *E. coli* strains NEB® 10-beta (New England Biolabs, Beverly, MA) for pCR™ 2.1-TOPO® plasmids, or λpir for pVSV105

plasmids, and XL10-Gold Ultracompetent Cells for point mutant mutagenesis (Agilent Technologies). Restriction enzymes (New England Biolabs, Beverly, MA) and T4 DNA Ligase (Invitrogen, Waltham, MA) were used for ligation reactions. Gel isolation, purification, and extraction of DNA were done by using Qiagen's QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Plasmid DNA was extracted using the Zyppy Plasmid Mini Prep kit (Zymo Research, Irvine, CA), and genomic DNA was isolated by phenol/chloroform extraction (Wilson, 2001).

II.c. Plasmid construction

All plasmids described herein are listed in Table 4. All primers mentioned are located in Table 5. Restriction enzyme pairs used in digests were heat inactivated before ligations following the manufacturer's protocol (New England Biolabs, Beverly, MA). If restriction enzymes were unable to be heat inactivated, they were column purified using a QIAquick kit (Qiagen, Valencia, CA). The gene variants described hereafter are depicted in Figure 4.

Gene VFMJ11_A0397 from MJ11 genomic DNA was amplified with primer pair "A0397 F SalI.1" and "A0397 RR trunc R1" at 57°C annealing, 1min 15sec elongation. The resulting amplicon was cloned into pCR™2.1-TOPO®, sequenced for 1x coverage at the UNH Hubbard Center for Genome Studies, and subsequently cloned into pVSV105 using restriction enzymes SalI and KpnI, generating plasmid pIMS1A4.

pIMS1A6 was generated by cloning an amplicon (gene VF_A0360) using primers “A0397 F SalI” and “A0360 R KpnI” at 56°C for 1min 30sec from ES114 genomic DNA into pCR™2.1-TOPO®. The resulting plasmid was sequenced for 1x coverage at the UNH Hubbard Center for Genome Studies, and cloned into pVSV105 using restriction enzymes SalI and KpnI, generating pIMS1A8.

pIMS1B1 was generated by amplifying the gene VFMJ11_A0397 using SOE PCR first with “A0397 F SalI.1” and “pA0397:REC SOE A R1”, and “A0397 R KpnI" and "pA0397:REC SOE B F1” at 50°C annealing for 20sec elongation, from MJ11 genomic DNA as template. After purification, the SOE PCR fragments were fused with “A0397 F SalI” and “A0397 R KpnI” by annealing at 50°C for 1min using Expand Long Template, purified, and cloned into pCR™2.1-TOPO®. The P_{binK}:REC amplicon was cloned from TOPO into pVSV105 using restriction enzymes SalI and KpnI, generating pIMS1B3.

pIMS1B6 was generated by first amplifying the MJ11 *qrr1* promoter using modified primers, “p16 IS” and “p17 IS” (Miyashiro et al. 2010), with added XmaI and XbaI restriction sites, respectively, into pCR™2.1-TOPO®. The *qrr1* promoter was then cloned into pTM267 upstream of *gfp* (Miyashiro et al. 2010) using restriction enzymes XmaI and XbaI, replacing the Kanamycin resistance gene, generating pIMS1B7.

pIMS1C1 was generated by first using site directed mutagenesis with primers “ebink quikH362Q A IS” and “ebink quikH362Q B IS”, and pIMS1C5 with primers “ebink quikD794A A IS” and “ebink quikD794A B IS” on pIMS1A6 as template, following manufacturer’s protocol, to

generate the point mutants of the ES114 BinK, H362Q and D794A, in pCR™2.1-TOPO®, respectively. pIMS1C9 was generated as above but with primers “ebink quikH362Q A IS” and “ebink quikH362Q B IS” on pIMS1C5 as template, generating pCR™2.1-TOPO® with the BinK double point mutant H362Q/D794A. The point mutant alleles H362Q, D796A, H362Q/D794A, were cloned from TOPO into pVSV105 with enzymes SalI and KpnI, generating plasmids pIMS1C3, pIMS1C5, and pIMS1D3, respectively. The two point mutant positions – H362Q and D794A – were identified based on NCBI annotations suggesting conserved sites of phosphorylation.

pIMS1D4 was generated by cloning gene VF_A0360, amplified with primers “IS ebink F SalI” and “IS ebink Δrec R KpnI” from ES114 genomic DNA at 51°C for 2min 37sec, into pCR™2.1-TOPO®. The resulting $BinK^{\Delta REC}$ was then cloned into pVSV105 with SalI and KpnI restriction enzymes, generating plasmid pIMS1D6.

pIMS1E5 was generated similar to pIMS1A4 but using MJ11EP2-4-1 as template.

II.d. Luminescence assays

Flasks with 10mL SWTO or SWT media were inoculating with 10 colonies of *V. fischeri* ES114 and MJ11 strain variants, respectively. After cultures reached an optical density (OD_{600}) of approximately 1.0, time points were measured every 20min for OD_{600} with 100µL culture into 500µL media blanks (or 200µL into 800µL for ES114 unless otherwise stated) with a D30 BioPhotometer (Eppendorf) then reading luminescence, by placing the same cuvette immediately

into a TD-20/20 luminometer (Turner Designs). For MJ11 strains, the culture was diluted up to 3,430x to achieve detectable luminescence, and for some ES114 strains, diluted 25x. Luminescence is reported as normalized luminescence, which is calculated by dividing the relative luminescence units (RLU; luminescence mL^{-1}/OD_{600}) of each replicate culture by the RLU of the wild-type harboring empty vector from the same experiment. Two pseudoreplicates were recorded per flask in the analyses. Statistical tests were performed on the RLU values using the approximate two-sample Fisher-Pitman permutation test with the default Holm correction to correct for false positive significance from multiple corrections, in the R package "coin".

II.e. Qrr1 expression assay

One colony each of *V. fischeri* ES114 or MJ11 strain harboring either pTM268 (for ES114; Miyashiro et al., 2010), pIMS1B7 (for MJ11) or pTM267 (empty vector) was inoculated from an LBS Cm agar plate into 100µL minimal medium in a flat black, clear bottom, 96 well microtiter plate (Costar). During incubation at 28°C, the OD_{600}, GFP fluorescence (excitation 485-nm, emission 535-nm) and mCherry fluorescence (excitation 535-nm, emission 612-nm) as reported (Miyashiro et al., 2010) was measured every hour for 45 hrs using an Infinite M200 plate reader (Tecan, Switzerland). Threshold fluorescence detection was determined empirically using wild-type bacteria harboring pVSV105 (plasmid without GFP or mCherry). Gain settings of 130 (GFP) and 160 (mCherry) were determined from pilot experiments to ensure fluorescence levels were above the detection threshold throughout kinetic cycles. The normalized fluorescence of the strains harboring either pTM268 or pIMS1B7 were evaluated against the same strain harboring the empty vector plasmid (pTM267), and between strains harboring pTM268 or

pIMS1B7, for significance using the approximate two-sample Fisher-Pitman permutation test with the default Holm correction to correct for false positive significance from multiple corrections, in the R package “coin”.

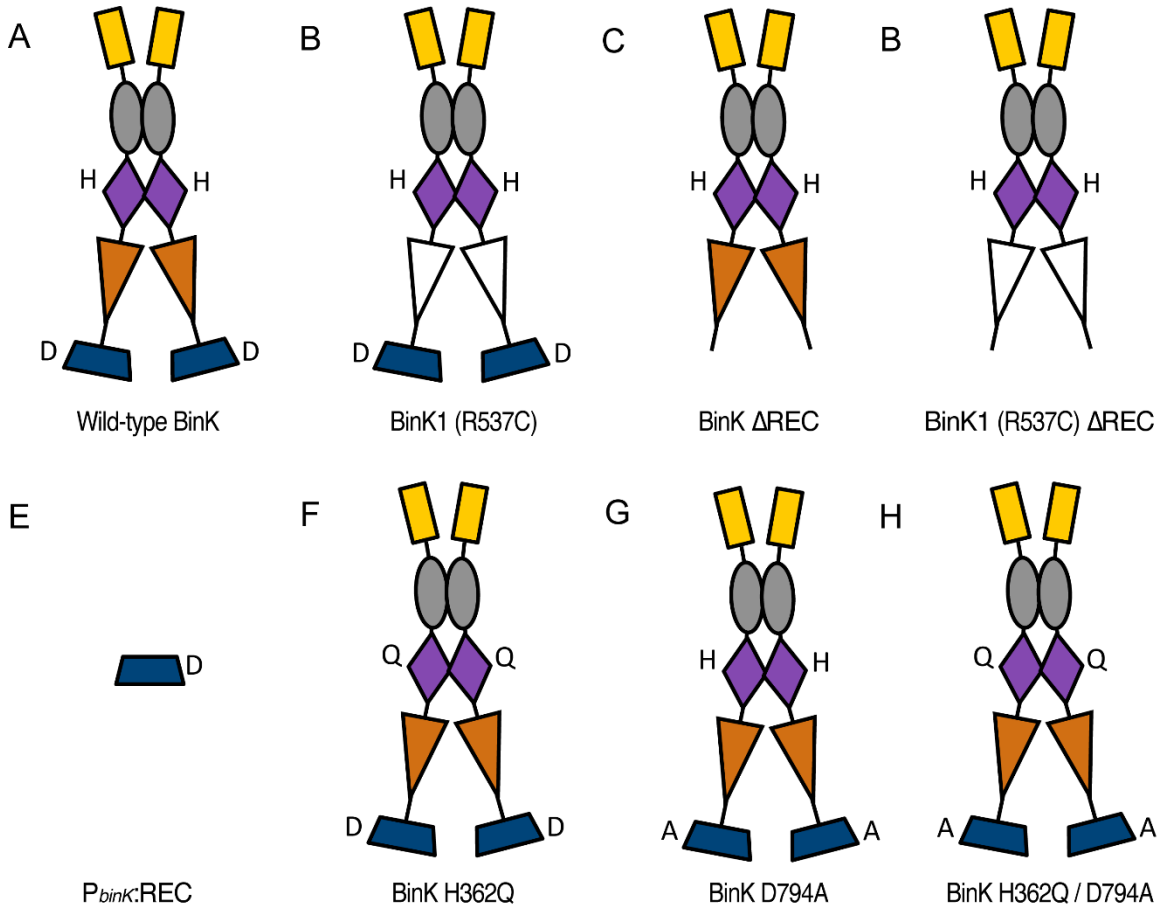


Figure 4. BinK allele variants depicted as homodimers.

Shapes: Yellow rectangle: Cache_1 domain; Gray oval: HAMP domain; Purple diamond: HisKA domain with histidine (H) or glutamine (Q) residue; Triangle: HATPase_C domain (Orange: wild-type HATPase; White: R537C mutation); Blue trapezoid: Receiver (REC) domain with aspartate (D) or alanine (A) residue.

Table 2. *Vibrio fischeri* strains used in this study.

Strain	Description	Source
ES114	Isolated from *Euprymna scolopes*	(Boettcher & Ruby, 1990)
CL42	ES114 *luxO::kanR*	(Lupp et al., 2003)
CL59	ES114 *luxOD47E**	(Lupp & Ruby, 2005)
KV1421	*att*Tn7::Erm	(O'Shea et al., 2006)
KV1548	VF2120 (*arcA*)	(Hussa et al., 2007)
KV1585	VF1570 (*torR*)	(Hussa et al., 2007)
KV1593	VFA0179	(Hussa et al., 2007)
KV1594	VF1401	(Hussa et al., 2007)
KV1595	VF1396 (*phoP*)	(Hussa et al., 2007)
KV1596	VFA0561	(Hussa et al., 2007)
KV1612	VFA1017	(Hussa et al., 2007)
KV1640	VFA0041 (*uhpA*)	(Hussa et al., 2007)
KV1641	VF1054	(Hussa et al., 2007)
KV1650	VFA0266	(Hussa et al., 2007)
KV1651	VF1988 (*phoB*)	(Hussa et al., 2007)
KV1654	VFA1012	(Hussa et al., 2007)
KV1655	VF2343 (*cpxR*)	(Hussa et al., 2007)
KV1665	VF1909 (*narP*)	(Hussa et al., 2007)
KV1668	VFA0211	(Hussa et al., 2007)
KV1672	VFA0181	(Hussa et al., 2007)
KV1714	VFA0795	(Hussa et al., 2007)
KV1715	VF0454 (*vpsR*)	(Hussa et al., 2007)
KV1727	VF0526 (*phoP*)	(Hussa et al., 2007)
KV1730	VF0095 (*ntrC*)	(Hussa et al., 2007)
KV1787	*ΔsypG*	(Hussa et al., 2007)
KV1809	VF1854 (*flrC*)	(Hussa et al., 2007)
KV2164	VF2374	(Hussa et al., 2007)
KV2165	VFA0216	(Hussa et al., 2007)
KV2501	VF1689 (*expM*)	(Hussa et al., 2007)
KV2503	VFA0103	(Hussa et al., 2007)
KV2505	VFA0802 (*cheV*)	(Hussa et al., 2007)
KV2507	VF0114 (*ompR*)	(Hussa et al., 2007)
KV2509	VFA0698 (*cheV*)	(Hussa et al., 2007)
KV2510	VF1833 (*cheY*)	(Hussa et al., 2007)
KV2636	VF1148 (*yehT*)	(Hussa et al., 2007)
KV2637	VF1879 (*cheV*)	(Hussa et al., 2007)
KV2874	VFA0732	(Hussa et al., 2007)
KV3299	*ΔsypE*	(Hussa et al., 2008)
KV4829	*ΔluxU*	(Miyashiro et al., 2014)
KV5972	*ΔluxQ*	(Miyashiro et al., 2014)
KV6010	*ΔluxP*	(Miyashiro et al., 2014)
KV7860	*ΔbinK*	K. Visick
MJ11	Isolated from *Monocentris japonica*	(Haygood et al., 1984)
MJ11EP2-4-1	MJ11 *BinK1*	(Pankey, Foxall et al., 2017)
RF1A4	MJ11 Δ*binK*::*ermR*; Erm^R	(Pankey, Foxall et al., 2017)

Abbreviations: Erm: Erythromycin; Kan: Kanamycin

* luxOD47E: LuxO protein with a glutamate (D) to aspartate (E) substitution at position 47

Table 3. *Escherichia coli* strains used in this study.

Strain	Description	Source
DH5α	endA1 hsdR17 (rK^- mK^+) glnV44 thi-1 recA1 gyrA (Nal^t) relA Δ(lacIZYA-argF)U169 deoR [φ80dlacΔ(lacZ)M15]	Gibco-BRL
DH5α λ*pir*	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE recA λpir* ; Amp^R	(Kolterand & Helinski, 1978)
NEB® 10-beta	Δ(*ara-leu*)*7697 araD139 fhuA* Δ*lacX74 galK16 galE15 e14-* Φ80*dlacZ*Δ*M15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1* Δ(*mrr-hsdRMS-mcrBC*)	New England Biolabs, Ipswich, MA
One-Shot TOP10	F- *mcrA* Δ(*mrr-hsdRMS-mcr*BC) Φ80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen, Carlsbad, CA
XL10-Gold	TetrD(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F′ proAB lacIqZDM15 Tn10 (TetR) Amy CmR]	Agilent Technologies

Abbreviations: Erm: Erythromycin; Str: Streptomycin; Tet: Tetracycline; Cm: Chloramphenicol; Kan: Kanamycin; Nal: Naladixic acid; Amp: Ampicillin

Table 4. Plasmids used in this study.

Plasmids	Description	Source
pCR™2.1TOPO®	Commercial cloning vector; Amp^R Kan^R	Invitrogen, Carlsbad, CA
pEVS104	Conjugal helper plasmid; *tra*$^+$ *trb*$^+$; Kan^R	(Stabb & Ruby, 2002)
pIMS1A4	pVSV105 carrying MJ11 *binKΔREC(ΔV747-T864)*	This study
pIMS1A6	pCR™2.1-TOPO® carrying ES114 wild-type *binK*	This study
pIMS1A8	pVSV105 carrying ES114 wild-type *binK*	This study
pIMS1B1	pCR™2.1-TOPO® carrying MJ11 P_{binK}*:REC (binK N737-T864)*	This study
pIMS1B3	pVSV105 carrying MJ11 P_{binK}*: REC (binK N737-T864)*	This study
pIMS1B6	pCR™2.1-TOPO® carrying MJ11 *qrr1* promoter	This study
pIMS1B7	pTM267 carrying MJ11 *qrr1* promoter	This study
pIMS1C1	pCR™2.1-TOPO® carrying ES114 *binK* C1086A (H362Q)	This study
pIMS1C3	pVSV105 carrying ES114 *binK* C1086A (H362Q)	This study
pIMS1C5	pCR™2.1-TOPO® carrying ES114 *binK* A2381C (D794A)	This study
pIMS1C7	pVSV105 carrying ES114 *binK* A2381C (D794A)	This study
pIMS1C9	pCR™2.1-TOPO® carrying ES114 *binK* C1086A/A2381C (H362Q/D794A)	This study
pIMS1D3	pVSV105 carrying ES114 *binK* C1086A/A2381C (H362Q/D794A)	This study
pIMS1D4	pCR™2.1-TOPO® carrying ES114 *binKΔREC (ΔK742-T864)*	This study
pIMS1D6	pVSV105 carrying ES114 *binKΔREC (ΔK742-T864)*	This study
pIMS1E5	pVSV105 carrying MJ11 *binK1 ΔREC (ΔV747-T864)*	This study
pRAD2E1	pVSV105 carrying MJ11 wild-type *binK*	(Pankey, Foxall et al., 2017)
pRF2A2	pVSV105 carrying MJ11 *binK1*	(Pankey, Foxall et al., 2017)
pTM267	pVSV105 carrying *Kan*^{R}*-gfp + PtetA-mCherry*; Cm^R	(Miyashiro et al., 2010)
pTM268	pVSV105 carrying ES114 P_{qrr1}*-gfp* and P_{tetA}*-mCherry*	(Miyashiro et al., 2010)
pVSV105	Mobilizable vector; Cm^R	(Dunn et al., 2006)

Abbreviations: Erm: Erythromycin; Cm: Chloramphenicol; Kan: Kanamycin

Table 5. Primer oligonucleotides used in this study.

Primer	Sequence (5' → 3')*	Source
A0397 F SalI.1	ATAAAGTCGACAAATGACGGATGTGTATGTGAGC	This study
A0397 F SalI	TCGACAAATAGAAACACTAACCAC	(Pankey, Foxall et al., 2017)
A0397 RR trunc R1	CTACTACAAGAACCGTTTTTATTATCTCTA	(Pankey, Foxall et al., 2017)
A0360 R KpnI	GGTACCGACCTAAACTAACAACCAT	This study
pA0397:REC SOE A R1	ACCGTTTTTATTATCTCTAGATTCATAAAAAACCTAGCACTT	This study
A0397 R KpnI	TAAAAGGTACCGAAATTAACGACCATTGATTACCC	(Pankey, Foxall et al., 2017)
pA0397:REC SOE B F1	AAGTGCTAGGTTTTTTATGAATCTAGAGATAATAAAAACGGT	This study
p16_IS	CCCGGGAGCCAAGACATCAAAACCTG	This study
P17_IS	TTTTTTCTAGAGGTCAATATACCTATTGCAGGG	This study
IS ebink F SalI	GTCGACCCAAAACGCTTATCCAAA	This study
IS ebinK deltaREC R KpnI	GGTACCTTATATTATGTCCAGATTATCCTTACG	This study
ebink quikH362Q A IS	AGTTCATTTTTAGCTAATATGTCACAAGAAATTCGAACACCTCTAAATGGCA	This study
ebink quikH362Q B IS	TGCCATTTAGAGGTGTTCGAATTTCTTGTGACATATTAGCTAAAAATGAACT	This study
ebink quikD794A A IS	ACCTTATAGTTTAGTTTTAATGGCCTGTATGATGCCGATAATGGATGGA	This study
ebink quikD794A B IS	TCCATCCATTATCGGCATCATACAGGCCATTAAAACTAAACTATAAGGT	This study
IS ebink SoeA R1	CCGTTTTTATTATGTCCAGATTCATAAAAAACCTAGCACTT	This study
IS ebink SoeB F1	AAGTGCTAGGTTTTTTATGAATCTGGACATAATAAAAACGG	This study
IS ebink R KpnI	GGTACCTCTACACCCTAAACTAACAACC	This study

*Underlined nucleotides indicate restriction enzyme sites within primers.

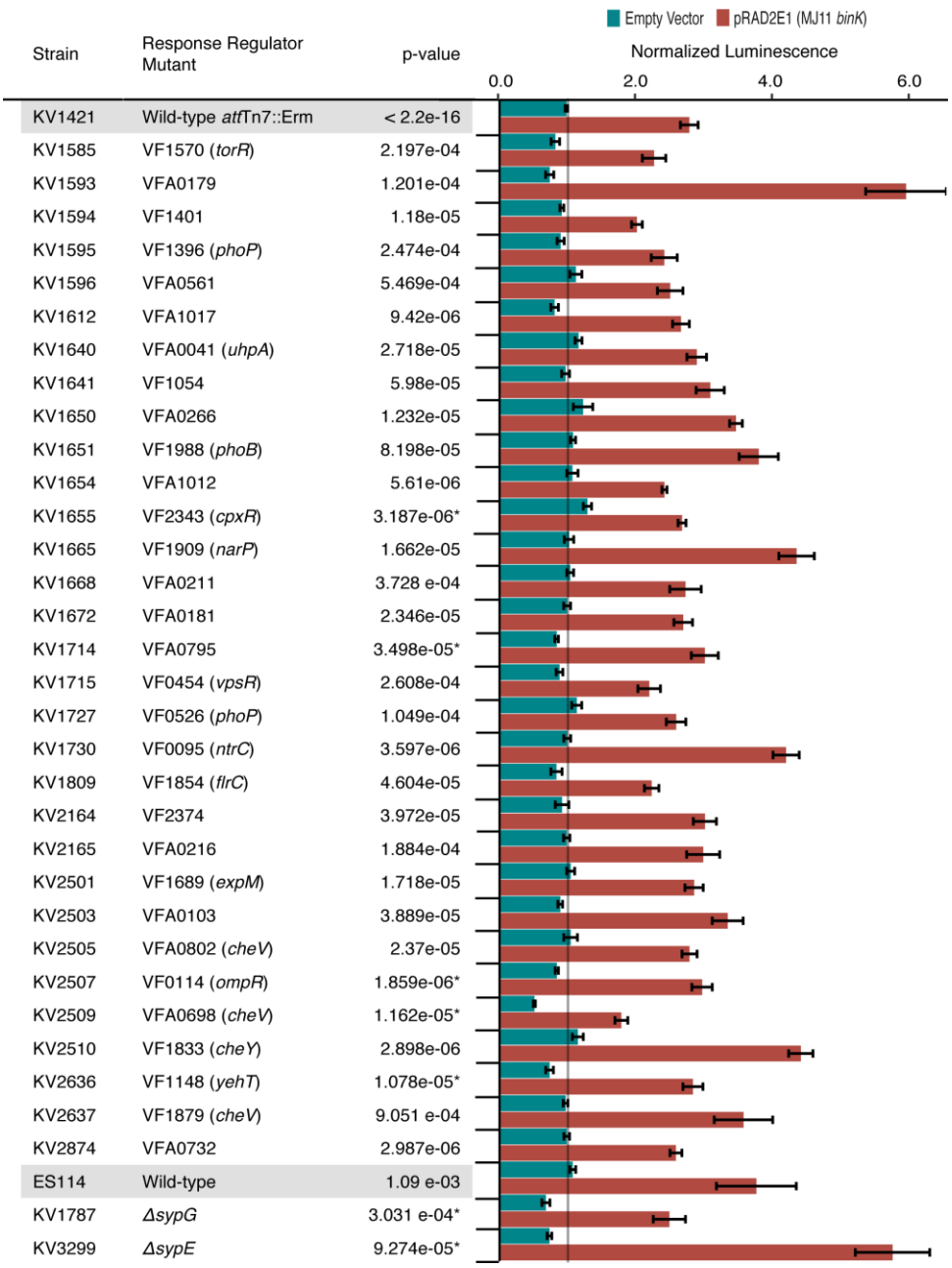
III. RESULTS

III.a. BinK does not require any orphaned RR to activate luminescence

The BinK hybrid HK is an unpaired orphan in that it has no cognate RR in close proximity, or co-transcribed, as is common for STS partners (Hussa et al., 2007); thus, the identity of its signaling partner(s) is not readily apparent. The genomes of both strain MJ11, the subject of our experimental evolution leading to discovery of BinK as a regulator of luminescence, and the native squid symbiotic strain ES114, have the same predicted complement of 40 RR proteins, some well-characterized, but many more poorly characterized and only 10 suggested as unpaired orphans (Hussa et al., 2007), among which could be a canonical partner for BinK. Null mutations in many of these RR were produced in the native symbiotic strain ES114 for the purpose of measuring their effects on phenotypes important for symbiosis with *Euprymna scolopes* (including luminescence) and specifically for identifying the signaling partner of a key horizontally-acquired sensor kinase that regulates symbiotic polysaccharide: RscS (Geszvain & Visick, 2008; Hussa et al., 2007). Because we expect that multi-copy expression of *binK* would not increase the luminescence of a derivative strain lacking its RR phosphorelay partner, provided BinK activation of luminescence in ES114 and MJ11 mirrors one another, these ES114 mutants could be useful for identifying the partner RR for BinK. To evaluate whether BinK activation of luminescence is conserved in both strains, we measured the influence of multi-copy expression of *binK* orthologs from MJ11 and ES114 (pRAD2E1 and pIMS1A8, respectively) on luminescence production by wild-type ES114. The *binK* alleles from MJ11 and ES114 similarly increased luminescence over ES114 harboring an empty plasmid

vector (2.613 +/- 0.131 and 2.610 +/- 0.121- fold increase, respectively) indicating that strain ES114 may be used to characterize the signaling cascade for BinK.

We subsequently evaluated the impact of multi-copy *binK* expression from MJ11 on bioluminescence in the previously generated ES114 RR mutant derivatives used for identifying the RscS partner (Hussa et al., 2007). In each of these, multi-copy expression of the MJ11 *binK* allele significantly increased luminescence compared to the same derivative harboring an empty vector (Fig. 5). Multi-copy expression even increased luminescence of derivatives with reduced luminescence, which are the most likely partners since their phenotype is most similar to loss of *binK* (Hussa et al., 2007; Pankey, Foxall et al., 2017). Though four uncharacterized RRs remain untested due to unsuccessful attempts to generate null mutations at these loci (Hussa et al., 2007), these data suggest that none of these RRs, even the orphaned or luminescence regulating RRs, are needed for the activation of luminescence by BinK. Therefore, BinK may influence luminescence through a branched, non-canonical pathway by cross-talk phosphorelay with a STS domain protein, potentially one that uses a well-characterized RR not included in this analysis.



Strain	Response Regulator Mutant	p-value
KV1421	Wild-type *att*Tn7::Erm	< 2.2e-16
KV1585	VF1570 (*torR*)	2.197e-04
KV1593	VFA0179	1.201e-04
KV1594	VF1401	1.18e-05
KV1595	VF1396 (*phoP*)	2.474e-04
KV1596	VFA0561	5.469e-04
KV1612	VFA1017	9.42e-06
KV1640	VFA0041 (*uhpA*)	2.718e-05
KV1641	VF1054	5.98e-05
KV1650	VFA0266	1.232e-05
KV1651	VF1988 (*phoB*)	8.198e-05
KV1654	VFA1012	5.61e-06
KV1655	VF2343 (*cpxR*)	3.187e-06*
KV1665	VF1909 (*narP*)	1.662e-05
KV1668	VFA0211	3.728 e-04
KV1672	VFA0181	2.346e-05
KV1714	VFA0795	3.498e-05*
KV1715	VF0454 (*vpsR*)	2.608e-04
KV1727	VF0526 (*phoP*)	1.049e-04
KV1730	VF0095 (*ntrC*)	3.597e-06
KV1809	VF1854 (*flrC*)	4.604e-05
KV2164	VF2374	3.972e-05
KV2165	VFA0216	1.884e-04
KV2501	VF1689 (*expM*)	1.718e-05
KV2503	VFA0103	3.889e-05
KV2505	VFA0802 (*cheV*)	2.37e-05
KV2507	VF0114 (*ompR*)	1.859e-06*
KV2509	VFA0698 (*cheV*)	1.162e-05*
KV2510	VF1833 (*cheY*)	2.898e-06
KV2636	VF1148 (*yehT*)	1.078e-05*
KV2637	VF1879 (*cheV*)	9.051 e-04
KV2874	VFA0732	2.987e-06
ES114	Wild-type	1.09 e-03
KV1787	*ΔsypG*	3.031 e-04*
KV3299	*ΔsypE*	9.274e-05*

Figure 5. Initial screen of *binK* in *trans* in known *Vibrio fischeri* response regulator mutants.

Luminescence of each strain harboring either an empty vector (pVSV105) or vector with *binK* (pRAD2E1) is reported as the normalized luminescence of the relative luminescence units (RLU; luminescence mL^{-1}/OD_{600}) of each replicate culture divided by the RLU of the wild-type harboring empty vector from the same experiment. The ability of multi-copy expression of *binK* (pRAD2E1) to significantly increase luminescence (*; $p<0.05$) of each variant (red bars) was determined by comparison to the same variant harboring empty vector (teal bars) by a two-way t-test in R. N=2 flasks from one experiment; N=8 for control KV1421; 1 flask per experiment. ES114 was for the wild-type reference for strains KV1787 and KV3299 and KV1421 (*attn*Tn7:erm) was the reference for all others. No difference is reported between ES114 or KV1421 harboring pVSV105 (p=0.07949; N=2 flasks from 2 experiments). Error bars are standard error.

III.b. BinK activates luminescence through the LuxO RR

Among the well-characterized RRs is the protein LuxO that integrates signaling initiated by two convergent quorum sensing pathways leading to activation of luminescence in response to increased cell density (Miyamoto et al., 2000). LuxO activity as a repressor of luminescence depends on its phosphorylation state, which is most directly influenced by availability of a high energy phosphate on its substrate partner HPt protein LuxU, which depends on the dual kinase and phosphatase activities of partner hybrid HKs: AinR and LuxQ/P (Lupp & Ruby, 2004; Lupp et al., 2003; Miyashiro & Ruby, 2012) (Fig. 3A-C). Though these two pathways converge onto the LuxU-LuxO signaling node and are thought to be insulated, proteins that are not homologs of AinR or LuxP/Q signal to the LuxU-LuxO node independently of AinR or LuxP/Q homologs in related *Vibrio* spp. (Jung et al., 2015). Therefore we considered whether BinK could activate luminescence via this quorum sensing pathway.

To evaluate whether BinK interfaces with the quorum sensing regulatory pathway through *trans* phosphorylation of the hybrid HKs, we first evaluated whether BinK was likely to heterodimerize with either AinR or LuxQ. Although differences in the AinR and BinK dimerization domain residues suggest they would not form a heterodimer, there was some conservation between LuxQ and BinK suggesting heterodimerization could be possible. Therefore, we used multi-copy expression of *binK* orthologs from MJ11 (pRAD2E1) and ES114 (pIMS1A8) in ES114 derivatives with mutations in *luxQ*, and *luxP*, which encodes the AHL accepting protein that binds to the sensory domain of LuxQ, to determine if these proteins are necessary for BinK activation of luminescence. Multi-copy expression of the MJ11 and ES114 *binK* alleles significantly increased the luminescence of *luxP* and *luxQ* mutant derivatives

(p=0.000, Fig. 6). These data indicate BinK does not branch to the quorum regulatory hybrid HK proteins to activate luminescence.

Next we evaluated whether LuxO was required for signaling by BinK. In contrast with results obtained with *luxP* and *luxQ* mutant derivatives (Fig. 6), multi-copy expression of the *binK* allele from MJ11 and ES114 did not increase the luminescence of a *luxO* mutant, which emulates a non-phosphorylated LuxO where luminescence repression is off (p=0.133, p=0.089, respectively). Consistent with this finding, multi-copy expression of the MJ11 *binK* allele in a lux^{OD47E} derivative that mimics LuxO~P where repression of luminescence is on, also did not increase luminescence (p=0.185). However, though relatively dim similar to the empty vector, the ES114 *binK* allele slightly increased luminescence (p=0.000). Thus, BinK requires a functional LuxO that can undergo de-phosphorylation in order to increase luminescence.

Because LuxU is the HPt that ordinarily donates the phosphate to LuxO, we next evaluated whether LuxU was required for BinK activation of luminescence. Curiously, both the *binK* alleles from MJ11 and ES114 modestly increased luminescence in a *luxU* mutant (p=0.029, p=0.031, respectively; Fig. 6). However, the significant increase in luminescence is not as pronounced compared to multi-copy expression of both alleles in the ES114 wild-type for *luxQ* or *luxP* mutant backgrounds, suggesting LuxU is needed for some of the increase in luminescence by *binK*. Although the identity of the interacting partner for BinK (LuxU, LuxO or both) is unresolved, these data suggest that BinK signaling branches to the LuxU-LuxO phosphorelay node independently of the upstream hybrid HKs to activate luminescence.

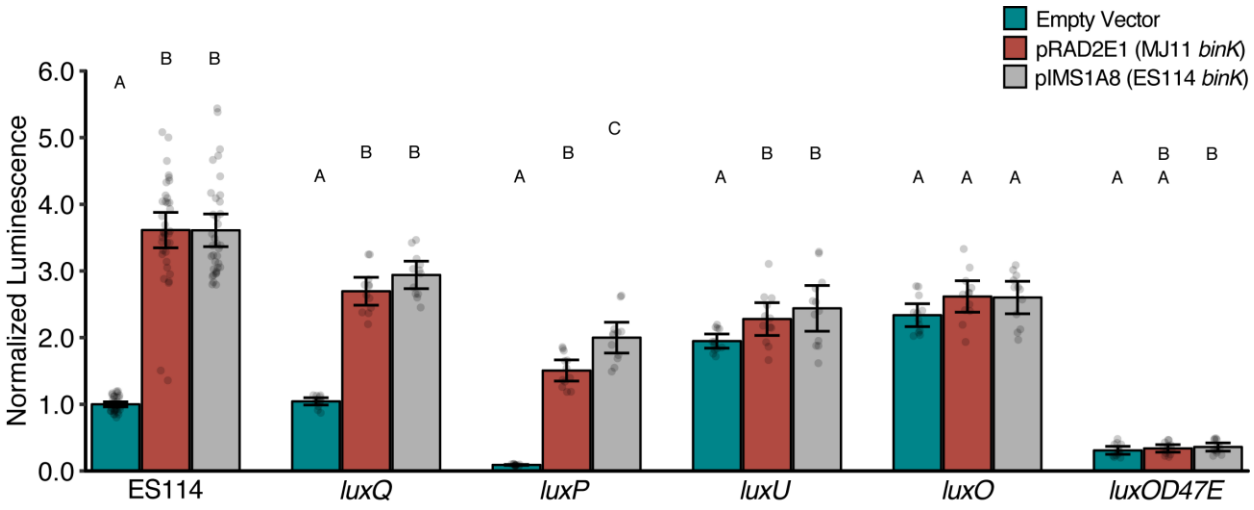


Figure 6. Effect of *binK* on maximum luminescence in ES114 QS regulation mutants.

Luminescence of each strain harboring either an empty vector (pVSV105) or vector with *binK* (pRAD2E1 or pIMS1A8) is reported as the normalized luminescence of the relative luminescence units (RLU; luminescence mL^{-1}/OD_{600}) of each replicate culture divided by the average RLU of the wild-type harboring empty vector from the same experiment. The increase in normalized luminescence by either the *binK* genes compared to the same strain harboring the empty vector was statistically evaluated using the approximate two-sample Fisher-Pitman permutation test with the default Holm correction to correct for false positive significance from multiple corrections, in the R package "coin". Each bar within their respective strain background not connected by letters are significantly different ($p<0.05$). Error bars are 95% confidence intervals. (N=6 flasks; 2 flasks of each strain with either plasmid across three experiments). The ES114 data is a combination of all controls that were done for all experiments (N=18 flasks). Shaded points indicate raw data where 2 pseudo replicates were recorded per flask.

III.c. The receiver (REC) domain of BinK is necessary for luminescence activation

As BinK contains domains that in other STS proteins have defined kinase/auto-phosphorylation or phosphatase/dephosphorylation activity, its interaction with the quorum sensing regulatory cascade at LuxU or LuxO could be mediated by individual domains rather than the complete protein as has been observed previously in branched pathways (Norsworthy & Visick, 2015). For instance the HisKA domain by itself can auto-phosphorylate by removing a phosphate from an interacting REC domain (such as in LuxO; Gao & Stock, 2009). Similarly, REC domains can auto-phosphorylate from a HPt domain (such as in LuxU) and also auto-dephosphorylate to release inorganic phosphate through its phosphatase activity, thereby restoring its auto-phosphorylation/phosphatase ability (Freeman et al., 2000), a mechanism used by HKs in STSs to return their RR partners to basal level activity. Notably, HPt domains exhibit neither kinase nor phosphatase activity but rather serve as inert substrates for phosphorelay. Thus, one can envision several potential interactions between BinK and either or both LuxU and LuxO, which would reduce the level of LuxO~P thereby activating luminescence.

To help disentangle which domains of BinK participate in luminescence regulation and whether BinK interacts with the HPt protein LuxU and/or the REC domain-containing protein LuxO, we first asked whether BinK utilizes its REC domain. Testing whether the REC domain is important will potentially point to the partner with which BinK interacts. If BinK requires its REC domain, it would suggest LuxU is the interacting partner whereas if the REC domain is not required, it would suggest the possibility that the HisKA domain functions to dephosphorylate LuxO.

To test this, we generated a truncated MJ11 BinK allele, removing its REC domain (pIMS1A4; $BinK^{\Delta REC}$), and also a construct wherein the REC domain alone is expressed in-

frame from the native BinK promoter and ribosomal binding site (pIMS1B3; P_{binK}:REC). We then evaluated whether multi-copy expression of these constructs enhanced the bioluminescence of ES114. $BinK^{\Delta REC}$ did not increase luminescence of ES114 (p=0.156), similar to the AinR homolog LuxN (Freeman et al., 2000), whereas P_{binK}:REC alone increased luminescence (p=0.000), although not to the extent that wild-type BinK increased luminescence (p=0.000; Fig. 7). Multi-copy expression of the $BinK^{\Delta REC}$ construct, as expected, did not increase luminescence of MJ11, but multi-copy expression of the REC domain unexpectedly also did not measurably enhance the luminescence of MJ11 (Fig. 7). Loss of BinK (*ΔbinK*) in MJ11 significantly decreased luminescence compared to wild-type (p=0.000), as was previously reported (Pankey, Foxall et al., 2017), and overexpression of P_{binK}:REC did increase luminescence of this dim mutant to a level that was comparable to wild-type (p=0.000). We surmise that the somewhat incongruent results could stem from the extremely bright and less consistent luminescence production of MJ11 and potential differences in sensitivity of perturbation, which complicates luminescence quantification. Therefore, further analysis of BinK luminescence regulation will focus more predominantly on the more experimentally amenable strain ES114. Regardless, these data suggest that BinK activates luminescence in a REC-dependent manner indicating that LuxU is a likely phosphorelay partner.

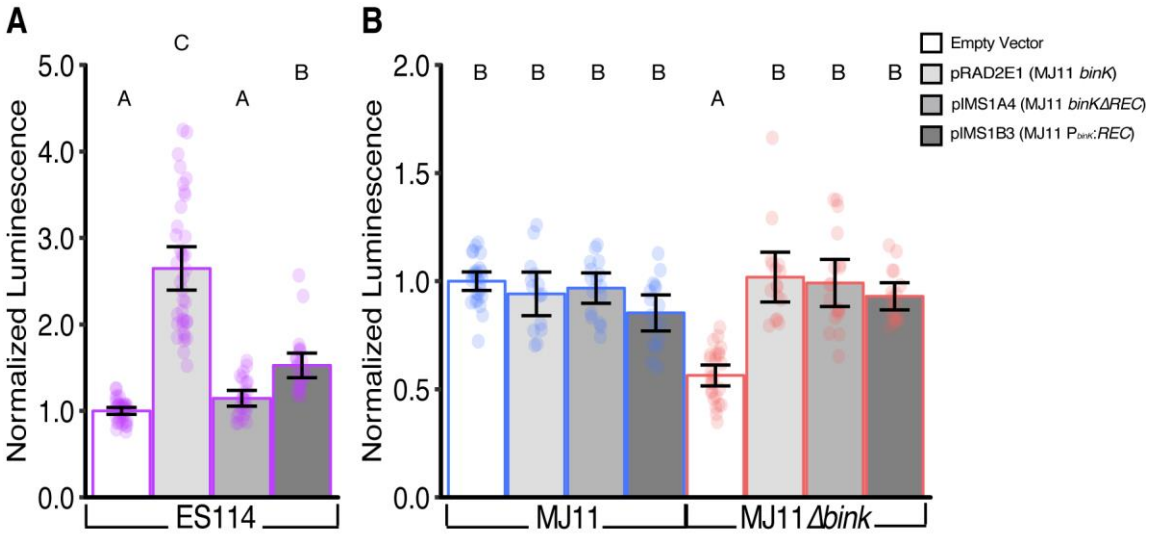


Figure 7. Effects of *binK* REC domain variants on maximum luminescence *Vibrio fischeri* strains.

Luminescence of each strain is reported as the normalized luminescence of the relative luminescence units (RLU; luminescence mL^{-1}/OD_{600}) of each replicate culture divided by the average RLU of the wild-type harboring empty vector (pVSV105) from the same experiment; either ES114 (A) or MJ11 (B). Each strain ES114 (purple), MJ11 (blue), and RF1A4 (MJ11 Δ*binK*, red) harbored either empty vector, empty vector with *binK* (pRAD2E1), empty vector with $binK^{\Delta REC}$ (pIMS1A4), and empty vector with P_{bink}:REC (pIMS1B3). The increase in normalized luminescence by either *binK* variant compared to the wild-type strain harboring the empty vector was statistically evaluated by using the approximate two-sample Fisher-Pitman permutation test with the default Holm correction to correct for false positive significance from multiple corrections, in the R package "coin". Each bar within their respective strain background not connected by letters are significantly different ($p<0.050$). Error bars are 95% confidence intervals. Each group of strains is combined from at least 3 experiments and each MJ11 strain variant experiment contained a MJ11 and Δ*binK* empty vector strain; in which the two are significantly different from one another ($p=0.000$). Shaded points indicate raw data where 2 pseudo replicates were recorded per flask.

III.d. The REC domain auto-phosphorylation residue but not the HisKA domain auto-phosphorylation residue is required for BinK activation of luminescence

Data thus far suggests that the BinK REC domain increases luminescence, similar to the AinR homolog LuxN, possibly by dephosphorylation of LuxU (Freeman et al., 2000). However, unlike with experiments with LuxN (Freeman et al., 2000), the BinK REC domain alone did not confer an increase in luminescence that was comparable to the wild-type protein (Fig. 6). Several explanations account for this ambiguity, including truncated protein instability or reduced domain activity due to differences in domain structure in the absence of the remainder of the BinK protein. To address these potential issues, we generated point mutations of the ES114 *binK* allele that changed the amino acid residues of the HisKA and REC domains that that undergo autophosphorylation. Specifically, to uncouple the activity of the two domains, we replaced the conserved HisKA-domain His residue that undergoes auto-phosphorylation (H362) with a glutamine residue, while maintaining function of the REC domain (Fig. 4F). In parallel, we replaced the conserved REC-domain auto-phosphorylating aspartate residue (D794) with an alanine residue, while maintaining function of the HisKA domain (Fig. 4G). Finally, we also generated a phosphorelay null allele, by combining both mutations (Fig. 4H).

The influence of domain-specific auto-phosphorylation mutations on bioluminescence of ES114 and its *ΔbinK* derivative was evaluated using multi-copy expression (Fig. 8). The HisKA-domain H362Q allele (pIMS1C3; $BinK^{H362Q}$) increased the luminescence of both ES114 and *ΔbinK* strains (p=0.000, Fig. 8), to a level comparable to that produced by over-expressing wild-type *binK* (p=1.000 and p=0.684 for ES114 and *ΔbinK*, respectively). This suggested that auto-phosphorylation by the HisKA domain, and specifically residue H362, plays little role in multi-copy enhancement of luminescence. In contrast, the D794A allele (pIMS1C7; $BinK^{D794A}$)

modestly decreased luminescence by ES114 (p=0.000) but did not change luminescence by the *ΔbinK* derivative (p=0.075, Fig. 8). As expected, the phosphorelay null H362Q/D794A (pIMS1D3; BinK$^{H362Q/D794A}$) did not increase luminescence of either wild-type ES114 or its *ΔbinK* variant (p=1.000 and p=0.115, respectively) and the ES114 BinK$^{\Delta REC}$ (pIMS1D6; BinK$^{ES114\Delta REC}$) allele also did not increase the luminescence of the *ΔbinK* variant (p=0.699), though modestly but significantly increased luminescence in the ES114 wild-type background (p=0.000), inconsistent with the MJ11 BinK$^{\Delta REC}$ in ES114 (Fig. 7). The inability of the BinK^{D794A} allele to enhance luminescence suggests BinK auto-dephosphorylation/phosphatase activity, and specifically the REC domain D794 residue, underlies BinK enhancement of luminescence.

The discovery of BinK as a regulator of luminescence was through a spontaneously arising mutant allele, *binK1*, that conferred a remarkable fitness gain during squid symbiosis (Pankey, Foxall et al., 2017). However, remarkably, the identified mutation R537C in *binK1* leading to its altered function and reduced luminescence mapped to the HATPase_C domain and not to the REC domain, suggesting this adaptive allele could have reduced activity. To test whether the mutation in *binK1* reduces luminescence activation independently of the REC domain or through an additive effect to affect phosphatase activity, we again employed multi-copy expression of the *binK1* allele without a REC domain (pIMS1E5; BinK1$^{\Delta REC}$). As previously shown (Pankey, Foxall et al., 2017), multi-copy expression of BinK1 increases luminescence by ES114 (p=0.000) but to a lesser extent compared to multi-copy expression of the wild-type BinK (p=0.000, Fig. 9). BinK1 increased luminescence to a level that was comparable to the increase conferred by REC domain alone (P$_{binK}$:REC). Finally, loss of the REC domain (BinK1$^{\Delta REC}$) eliminated the ability BinK1 to increase luminescence to the levels of

BinK1 (p=0.000, Fig. 9). This suggests that while BinK1 has a reduced ability to enhance luminescence, this activity still lies within its REC domain.

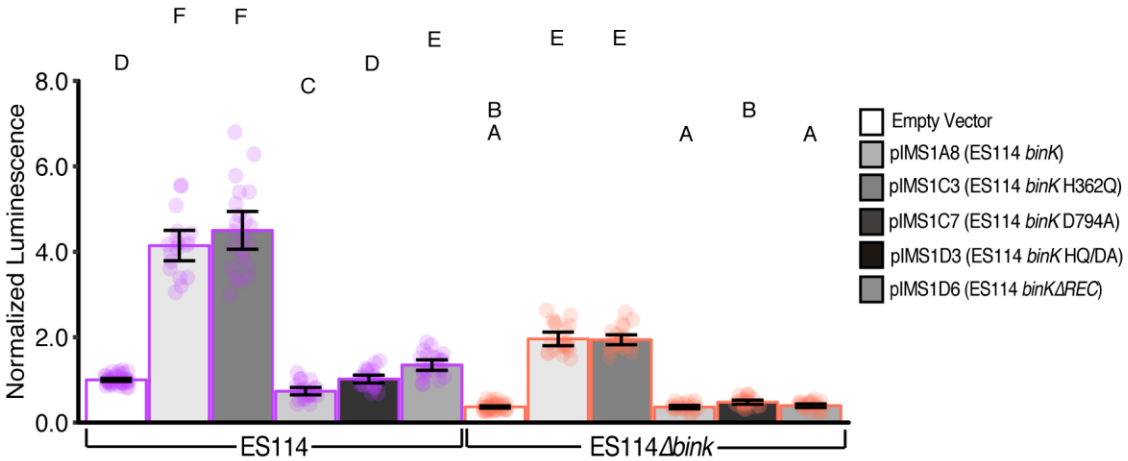


Figure 8. Maximum luminescence of *Vibrio fischeri* ES114 *binK* point mutation alleles in ES114 and ES114 *ΔbinK* backgrounds.

Luminescence of each strain harboring either an empty vector (pVSV105) or empty vector with a ES114 *binK* gene variant is reported as the normalized luminescence of the relative luminescence units (RLU; luminescence mL^{-1}/OD_{600}) of each replicate culture divided by the average RLU of the wild-type ES114 harboring empty vector from the same experiment. Left to right in each strain ES114 (purple) and KV7860 (ES114 Δ*binK*; orange): empty vector, empty vector with *binK* (pIMS1A8), empty vector with *binK* with point mutation H362Q (pIMS1C3), empty vector with *binK* with point mutation D794A (pIMS1C7), empty vector with *binK* with both point mutations H362Q/D794A (pIMS1D3), or empty vector with $binK^{\Delta REC}$ (pIMS1D6). The increase in normalized luminescence by either *binK* variant compared to the wild-type strain harboring the empty vector was statistically evaluated by using the approximate two-sample Fisher-Pitman permutation test with the default Holm correction to correct for false positive significance from multiple corrections, in the R package "coin". Each bar within their respective strain background not connected by letters are significantly different ($p<0.050$). Error bars are 95% confidence intervals. Each group of strains are a combination of four experiments (N=10 flasks across 4 experiments) and contained both ES114 and Δ*binK* empty vectors; which are significantly different from one another ($p=0.000$). Shaded points indicate raw data where 2 pseudo replicates were recorded per flask.

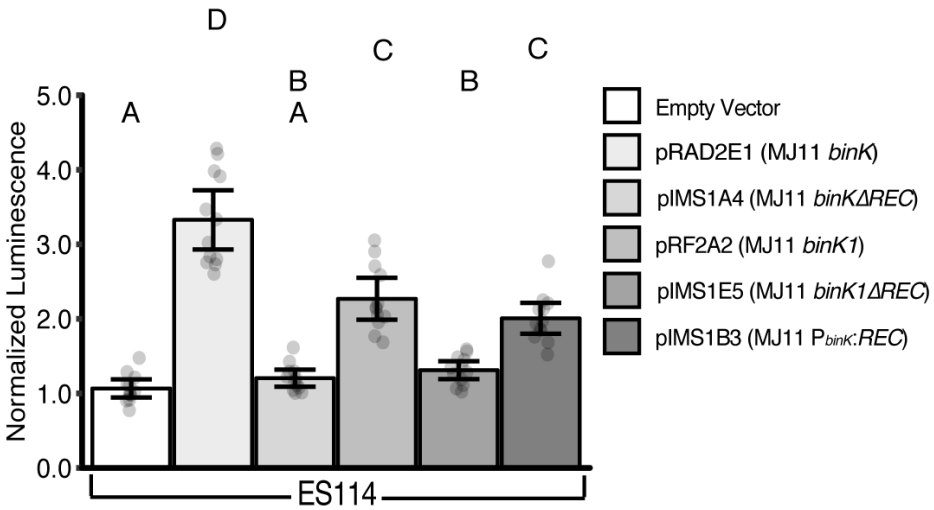


Figure 9. Maximum luminescence of *Vibrio fischeri* MJ11 *binK* and *binK1* REC domain variants in *V. fischeri* ES114.

Luminescence is reported as the normalized luminescence of the relative luminescence units (RLU; luminescence mL^{-1}/OD_{600}) of each replicate culture divided by the average RLU of the wild-type harboring empty vector (pVSV105) from the same experiment. Plasmids from left to right: empty vector, empty vector with *binK* (pRAD2E1), empty vector with $binK^{\Delta REC}$ (pIMS1A4), empty vector with *binK1* (pRF2A2), empty vector with $binK1^{\Delta REC}$ (pIMS1E5), and empty vector with P_{bink}:REC (pIMS1B3). The increase in normalized luminescence by either *binK* variant compared to the wild-type strain harboring the empty vector was statistically evaluated by using the approximate two-sample Fisher-Pitman permutation test with the default Holm correction to correct for false positive significance from multiple corrections, in the R package "coin". Each bar not connected by letters are significantly different ($p<0.050$). Error bars are 95% confidence intervals. Each plasmid was run in triplicate flasks across two experiments (N=6 flasks total). Shaded points indicate raw data where 2 pseudo replicates were recorded per flask.

III.e. ES114 *ΔbinK* and MJ11 *binK1* increase *qrr1* expression

These multi-copy expression data suggest that BinK, independently of the HisKA domain, uses the REC domain to influence levels of LuxO~P, potentially via LuxU as a non-canonical STS pathway. If our interpretation is correct, loss of *binK* (*ΔbinK*, RF1A4 for MJ11; KV7860 for ES114) or reduction of BinK activity (*binK1*; MJ11EP2-4-1) either of which reduces luminescence (Pankey, Foxall et al., 2017), should lead to higher levels of active LuxO~P which would result in increased production of the small RNA *qrr1* (Miyashiro et al., 2010; Pankey, Foxall et al., 2017). Using promoter fusion constructs where *gfp* expression is driven by either the ES114 *qrr1* promoter (pTM268) (Miyashiro et al., 2010) or the MJ11 *qrr1* promoter (pIMS1B7), we measured whether altered *binK* alleles resulted in elevated P_{qrr1}-*gfp*. Consistent with our proposed regulatory pathway, loss of *binK* (*ΔbinK*) increased *qrr1* expression for both MJ11 and ES114 (p=0.014, Fig. 10). The evolved *bink1* allele of strain MJ11, which attenuates quorum sensing and luminescence (Pankey, Foxall et al., 2017) also increased *qrr1* expression compared to both wild-type MJ11 and *ΔbinK* (p=0.014, Fig. 10). However, the *qrr1* expression of the MJ11 strains does not mimic what is expected based on autoinducer and luminescence levels. We hypothesized that *ΔbinK* would maintain a higher level of *qrr1* expression because *ΔbinK* is dimmer than *binK1* (Pankey, Foxall et al., 2017). The *qrr1* results in the ES114 background help confirm that BinK activates luminescence in a LuxO-dependent manner, affecting downstream *qrr1* expression. In MJ11 the *qrr1* expression was also enhanced in both mutants, and despite the unexpected difference in relation to *binK1* and *ΔbinK*, BinK in MJ11 is confirmed to work upstream of LuxO.

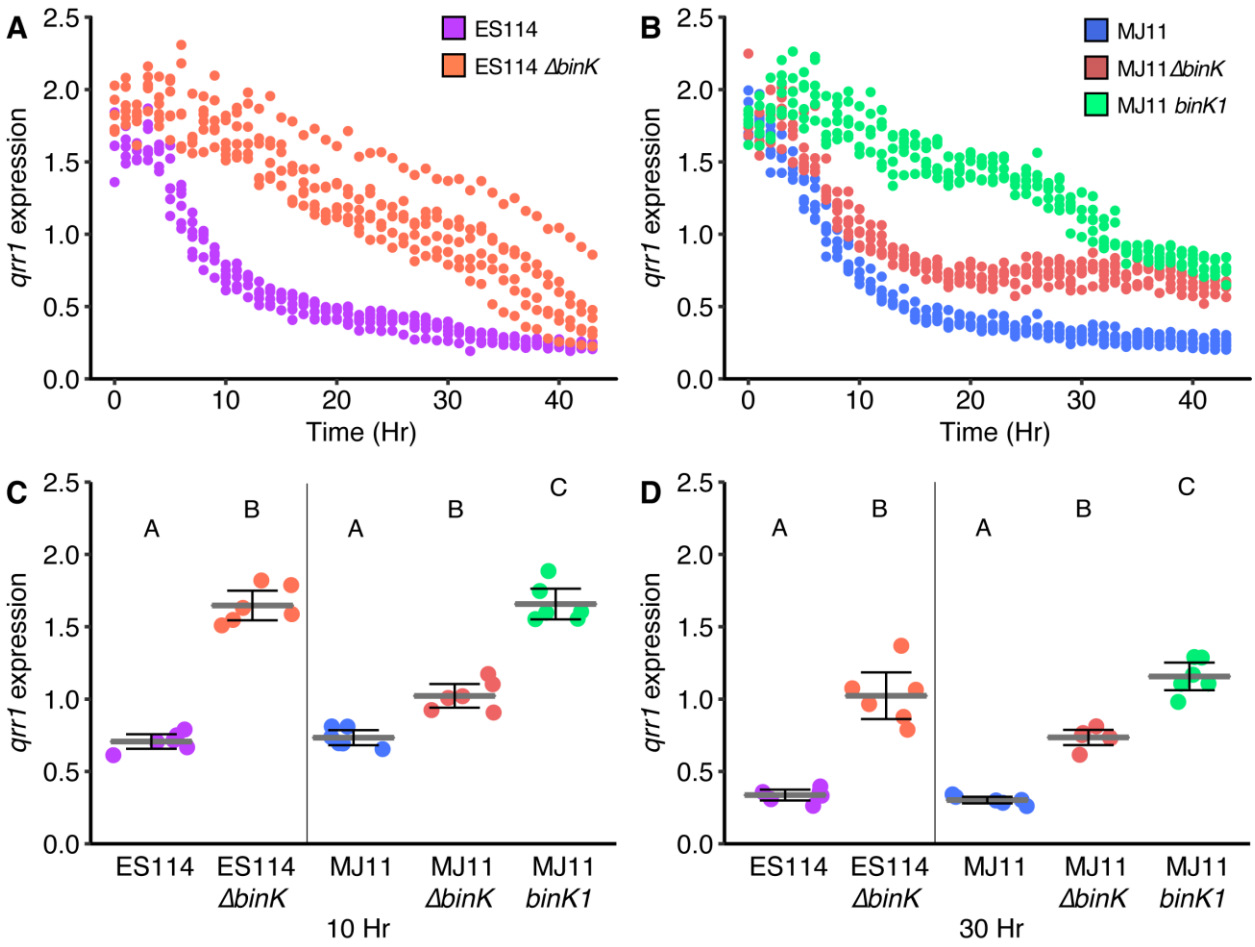


Figure 10. Expression of small RNA quorum sensing regulator *qrr1* in both *Vibrio fischeri* ES114 and MJ11 backgrounds.

Expression of *qrr1* was measured by dividing GFP expression (*qrr* promoter) by mCherry expression (*tetR* promoter) over time. **A)** Expression of *qrr1* by ES114 (purple) and KV7860 (ES114 Δ*binK*, orange) harboring *qrr1* promoter-fusion of ES114 (pTM268) and **B)** MJ11 (blue), RF1A4 (MJ11 Δ*binK*, red), and the evolved MJ11 *binK1* (MJ11EP2-4-1, green) harboring *qrr1* promoter-fusion of MJ11 (pIMS1B7). **C)** 10 hour time point and **D)** 30 hour time point from panel A and B time course. The amount of *qrr1* as expressed via the amount of GFP produced by the promoter-fusion constructs were statistically evaluated between each strain, ad against the same strains harboring empty vector (pTM267) using approximate two-sample Fisher-Pitman permutation test with the default Holm correction to correct for false positive significance from multiple corrections, in the R package "coin". Each bar within their respective strain background not connected by letters are significantly different ($p<0.050$). Error bars are 95% confidence intervals. All *qrr1* promoter-fusion plasmids for each strain background at both time points were significantly higher and removed from the graph. These data are a representative experiment (each strain with plasmid, n=6). Colored points indicate individual data.

III.f. The location of the BinK1 mutation in the HATPase_C domain is incongruous with the reduced ability of this allele for REC-dependent luminescence activation suggesting interactions between kinase and phosphatase domains are unresolved

BinK1 is a spontaneously-arising allele where the amino acid residue cysteine (C) replaced the arginine (R) at position 537 within the HATPase_C domain, the domain important for catalyzing phosphorylation of the conserved His residue within the HisKA domain (Pankey, Foxall et al., 2017). However, it is not intuitive how this mutation would influence REC domain activity leading to decreased luminescence activation (Pankey, Foxall et al., 2017). Furthermore, the higher expression of *qrr1* in this mutant compared to the null *ΔbinK* mutant in MJ11, is particularly unexpected given that the BinK1 variant produces more light than ΔBinK.

To glean insight into how this mutation could impact function, the BinK HATPase_C domain was aligned with domains from known HKs PhoQ and EnvZ. Aligning the domains localizes the residue in relation to known characterized features of the domain, (Marina et al., 2001) (Fig. 11), to determine whether the BinK1 mutation is an important functional residue. Indeed, the BinK1 R537C mutation is localized to the ATP lid that is important for binding of the γ-phosphate of the ATP molecule for phosphorylation catalysis (Marina et al., 2001). However mutating this residue in PhoQ (R439) does not affect ATP binding or catalysis kinetics compared to other mutations within the ATP lid, such as residue R434 (Fig. 11) which is important in binding the β-phosphate group of the ATP and is critical for catalytic function (Marina et al., 2001). At the position corresponding to this critical R434 residue, however, BinK contains a glutamine residue (Q530). Kinases with a glutamine in this position could have different catalytic mechanisms, while overall accomplishing the same role (Marina et al., 2001). This comparative alignment with PhoQ and EnvZ HKs and combined with the functional studies, could suggest that the BinK1 mutation has reduced kinase function.

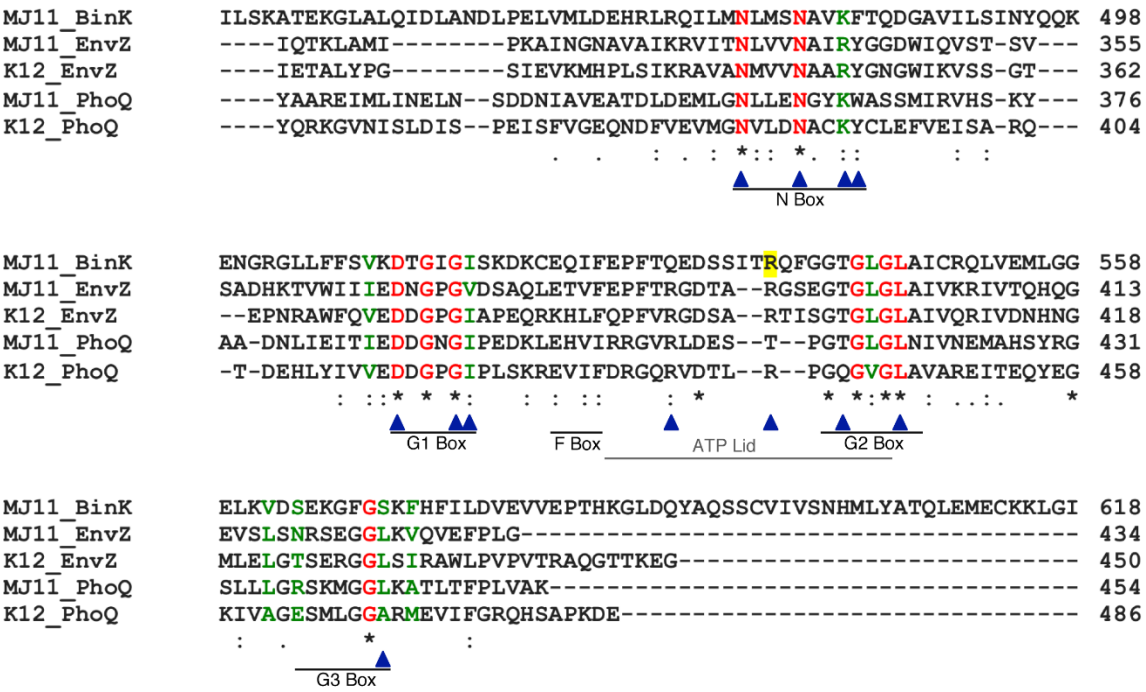


Figure 11. Sequence alignment of HATPase_C domain in *Vibrio fischeri* strain MJ11's BinK, EnvZ, PhoQ, and *Escherichia coli* strain K-12's EnvZ and PhoQ proteins.

Sequence alignment was performed in Clustal Omega with default parameters (Sievers et al., 2011). Characteristics of conserved regions within the domain are indicated by black underlines and blue triangles indicate residues important with the ATP nucleotide and Mg^{+2} ion binding as shown in (Marina et al., 2001) with red and green letters indicating ATP-binding residues as annotated by NCBI's MJ11 sequences where red are conserved and green are not conserved residues. BinK1 allele mutation position R537C is indicated by yellow highlight. All MJ11 sequences were downloaded from NCBI (BinK: ACH63581.1, EnvZ: ACH65053.1, PhoQ: ACH66645.1) and *E. coli* sequences from UniProt database (EnvZ: P0AEJ4, PhoQ: P23837 (Ikura et al., 1998).

IV. DISCUSSION

Quorum sensing bacteria integrate multiple specific signals to enable crucial group behaviors like biofilm and bioluminescence production that are used to associate with eukaryotic hosts (Basslet et al., 1993; Hammer & Bassler, 2003; Henke & Bassler, 2004a; Lupp & Ruby, 2005; Miyamoto et al., 2000; Ray & Visick, 2012; Visick et al., 2000; Zhu et al., 2001). These multiple sensors provide functional redundancy in quorum sensing regulation to stabilize cells in the presence of population cue disturbances (Jung et al., 2015). Currently, there have been no newly discovered quorum sensing regulators in *Vibrio fischeri*, aside from the hybrid HKs AinR and LuxQ, until recently when a single point mutation in the sensor kinase BinK (BinK1) altered bioluminescence production during an adaptive leap to symbiosis by strain MJ11 (Pankey, Foxall et al., 2017). Unlike the current sensory inputs used to regulate luminescence, BinK is orphaned in the genome, has no obvious canonical RR partner, and the mechanism behind how BinK mediates luminescence is unknown.

Unexpectedly, BinK acts similar to AinR and LuxQ through intrinsic REC-dependent phosphatase activity (Fig. 8), most likely to dephosphorylate LuxU and reduce LuxO~P repression of luminescence (Fig. 6). LuxU, an HPt protein, is the likely partner because REC domains, with a conserved aspartate (Asp) residue, initiate phosphotransfer between a conserved histidine (His) residue in the core HisKA kinase domain to a conserved His residue in an intermediate HPt protein. If BinK did not activate luminescence in a REC-dependent manner, LuxO would be a likely direct phosphorelay partner as HisKA domains interact with RR REC domains in the traditional two-component STS (Gao & Stock, 2009).

As an exception to canonical hybrid HK function where REC domains mediate transfer between the two conserved His residues, the REC domain of HKs can instead stabilize phosphotransfer between the conserved HisKA-His residue to the conserved Asp residue in the RR REC domain (Petters et al., 2012; Wise et al., 2010). BinK could directly interact with LuxO by using the REC domain as a stabilization factor, but the fact that the REC-Asp residue is critical for luminescence activation and the conserved His residue in the HisKA domain is not critical suggests this interaction is unlikely (Fig. 8). Additionally, BinK could interact with another unidentified HPt protein that subsequently interacts with LuxO, providing one explanation for the ability of BinK to modestly increase luminescence of the *luxU* mutant (Fig. 6). Though BinK could utilize other HPt proteins and domains within the *V. fischeri* genome (Randi Foxall, personal communication), due to the critical role of the LuxU – LuxO node in *V. fischeri* and in the other *Vibrio* spp for quorum sensing, LuxU is the most likely target (Henke & Bassler, 2004b; Jung et al., 2015).

Conclusions drawn from the multi-copy expression data that generated the current model of how BinK regulates bioluminescence must, however, be taken with caution. Multi-copy overexpression of a gene does not place the gene in the native single copy context and can produce results that are artifacts of gene dosage and do not reflect normal interactions. However, since the multi-copy expression of $BinK^{\Delta REC}$ and $BinK^{D794A}$ matched the luminescence produced by wild-type with an empty vector or in some cases slight significant but showing a similar trend, these data remain viable options for expected functional outcomes. To address these shortcomings, single copy genomic mutants could be generated to test the role of BinK in luminescence regulation in a more realistic single copy context. Since genomic deletions of *binK* in the ES114 and MJ11 strain backgrounds produce notable decreases in luminescence (Pankey,

Foxall et al., 2017) (Fig. 8), we anticipate that expression of *binK* alleles harboring point mutations will reproduce results observed with multi-copy expression.

Though the AinR and LuxQ homologs are extensively characterized biochemically, not much is known about how fine adjustments in the innate kinase / phosphatase activities affect luminescence activation. The naturally occurring *binK1* R537C point mutation is located in an important ATP binding pocket within the HATPase_C domain and not in a domain like the auto-phosphorylating HisKA domain or the auto-dephosphorylating REC domain that would more obviously lead to decreased de-phosphorylation ability (Fig. 11). Therefore this natural variant provides an unique opportunity to understand how the catalytic domain affects kinase and phosphatase activity within hybrid HKs (Pankey, Foxall et al., 2017).

BinK1 is hypothesized to operate in a REC-dependent manner with weakened or slowed phosphatase / dephosphorylation activity that keeps more phosphates on LuxU thereby delaying luminescence induction (Schuster et al., 2010), increasing *qrr1* expression (Fig. 10), and decreasing light production (Pankey, Foxall et al., 2017) (Fig. 9). However, from the multiple sequence alignment comparing known PhoQ and EnvZ HATPase domains we surmised that BinK1 has lowered kinase activity (Fig. 11). As demonstrated, inactivation of the HisKA-His residue that receives the phosphate from ATP does not impact luminescence activation by BinK, thus a mutation in the HATPase_C domain would not provide the gain of function seen by BinK1 if kinase activity by itself is important. Since PhoQ and EnvZ HKs lack a REC domain, and consequently its phosphatase / dephosphorylation function, which is preferred over the phosphorylation of the conserved His residues in hybrid HKs (Kinoshita-Kikuta et al., 2015), these proteins could have slight differences in how the HATPase_C domains regulate kinase and phosphatase functions. Congruent with the hypothesis of reduced phosphatase activity,

homologous hybrid HKs with a HATPase mutation enabled constitutive kinase and reduced phosphatase activity through an inability to switch between functional states (Kim et al., 2013; Wiesmann, 2016). If the R537C mutation hinders BinK1 to switch between functional states, or reduces the efficacy of the REC domain to fully utilize phosphatase activity, then BinK1 could partially lose the ability to activate luminescence. Though the mechanism of this mutation remains to be tested and its effect on kinase - autophosphorylation activity is unknown, these studies highlight the role HATPase domains might have on both auto-phosphorylation and – dephosphorylation activity that would have otherwise been missed from studying mutations produced in the conserved His and Asp residues.

Future work will enlist a biochemical approach to measure the phosphotransfer capabilities between the laboratory and naturally generated BinK point mutants to capture kinetic differences in the kinase and phosphatase functions. Although these phosphotransfers between His and Asp residues are rapidly turned over, incubating them with radiolabeled P32-γ-ATP and phosphoimaging will capture the kinetic profile of these proteins (Casino et al., 2014). These experiments will elucidate how BinK1 is able to reduce luminescence and overall provide insight into how hybrid HKs regulate kinase and phosphatase activities.

Quorum sensing depends on the reception of unique signals by sensor kinases to elicit appropriate responses. All known characterized sensor kinases that regulate quorum sensing in the *Vibrio* spp. respond to a specific signal (Henke & Bassler, 2004b; Jung et al., 2015; Lupp et al., 2003), except for the newly identified regulator BinK where there is no known signal. Perhaps quorum sensing regulation in *V. fischeri* is unique in which BinK acts as an internal regulator of symbiosis where it is not immediately needed to sense a population cue, thus only active in a phosphatase – on state. Although the purpose of BinK in quorum sensing is unclear,

this study further emphasizes the importance sensor kinases have in using dual kinase – phosphatase activities and why regulating these activities is crucial to these proteins serving as extra checkpoints in important cellular processes relevant to their lifestyles.

APPENDIX

Table 6. Abbreviations used in this study.

Abbreviation	Meaning
STS	Signal Transduction System
His / H	Histidine
Asp / D	Aspartate
RR	Response Regulator
REC	Receiver
HisKA	Histidine Kinase A
HATPase	Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase
HK	Histidine Kinase
HPt	Histidine Phosphotransferase
PAS	Per-Arnt_sim domain
HAMP	Histidine kinase, Adenyl cyclase, Methyl-carrier, and Phosphatase
HHK	Hybrid Histidine Kinase
Syp	Symbiotic polysaccharide
HSL	Homoserine Lactone
AI	Autoinducer
C8-HSL	*N*-octanoyl homoserine lactone
3OC6-HSL	*N*-3-oxo-hexanoyl homoserine lactone
Gln / Q	Glutamine
Ala / A	Alanine
Arg / R	Arginine
Cys / C	Cysteine
SWT	Seawater Tryptone
IO	Instant Ocean
HMM	HEPES minimal media
SOE	Splice Overlap Extension
Erm	Erythromycin
Kan	Kanamycin
Str	Streptomycin
Tet	Tetracycline
Cm	Chloramphenicol
Nal	Naladixic acid
Amp	Ampicillin
RLU	Relative Luminescence Units
GFP	Green Flourescent Protein

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