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Non-native acylated homoserine lactones reveal that LuxIR quorum sensing promotes symbiont stability

Sarah V. Studer,[1†¶] Julia A. Schwartzman,[1¶] Jessica S. Ho,[1‡] Grant D. Geske,[2§] Helen E. Blackwell[2] and Edward G. Ruby[1*]

Departments of [1]Medical Microbiology and Immunology and [2]Chemistry, University of Wisconsin-Madison, 1550 Linden Drive, Madison, WI 53706, USA.

Summary

Quorum sensing, a group behaviour coordinated by a diffusible pheromone signal and a cognate receptor, is typical of bacteria that form symbioses with plants and animals. LuxIR-type *N*-acyl L-homoserine (AHL) quorum sensing is common in Gram-negative *Proteobacteria*, and many members of this group have additional quorum-sensing networks. The bioluminescent symbiont *Vibrio fischeri* encodes two AHL signal synthases: AinS and LuxI. AinS-dependent quorum sensing converges with LuxI-dependent quorum sensing at the LuxR regulatory element. Both AinS- and LuxI-mediated signalling are required for efficient and persistent colonization of the squid host, *Euprymna scolopes*. The basis of the mutualism is symbiont bioluminescence, which is regulated by both LuxI- and AinS-dependent quorum sensing, and is essential for maintaining a colonization of the host. Here, we used chemical and genetic approaches to probe the dynamics of LuxI- and AinS-mediated regulation of bioluminescence during symbiosis. We demonstrate that both native AHLs and non-native AHL analogues can be used to non-invasively and specifically modulate induction of symbiotic bioluminescence via LuxI-dependent quorum sensing. Our data suggest that the first day of colonization, during which symbiont bioluminescence is induced by LuxIR, is a critical period that determines the stability of the *V. fischeri* population once symbiosis is established.

Received 12 July, 2013; accepted 29 October, 2013. *For correspondence. E-mail egruby@wisc.edu; Tel. (+1) 608 262 5911; Fax (+1) 608 262 8418. Present addresses: †Fuel Cell Technologies Office, Office of Energy Efficiency and Renewable Energy (EERE), Department of Energy, 1000 Independence Avenue SW, Washington, DC 20009, USA; ‡Laboratory of Immune Cell Epigenetics and Signaling, The Rockefeller University, 1230 York Avenue, New York, New York 10065, USA; §Cushing Academy, 39 School St., Ashburnham, MA, 01430, USA. ¶These authors contributed equally. Author contributions: SVS and JAS performed the experiments, and JAS performed the data analysis. JSH and GDG performed preliminary experiments. JSH, HEB and EGR helped with experimental design. JAS, SVS and EGR prepared the manuscript, with help from, and editing by, all co-authors.

Introduction

A common thread among microbes that initiate symbioses with plant and animal hosts is the quorum sensing-dependent regulation of colonization factors (Parsek and Greenberg, 2000; González and Venturi, 2012). Quorum sensing relies on perception of an endogenously synthesized secreted pheromone signal molecule, called an autoinducer, by a cognate receptor in a concentration-dependent manner. LuxIR quorum-sensing systems are widespread among Gram-negative bacteria, which use a LuxR-type quorum-sensing receptor to perceive an *N*-acyl L-homoserine lactone (AHL) chemical signal(s) (Churchill and Chen, 2010). The importance of AHL-based quorum sensing in pathogens such as *Pseudomonas aeruginosa*, or mutualists such as *Sinorhizobium meliloti* (Teplitski *et al.*, 2011; Galloway *et al.*, 2012), has led to significant interest in developing methods to manipulate this regulatory circuit *via* interception of the native AHL signal molecule (Rasmussen and Givskov, 2006; Amara *et al.*, 2011; Galloway *et al.*, 2011; Praneenararat *et al.*, 2012).

Despite this interest, only a few studies (Hentzer *et al.*, 2003; Wu *et al.*, 2004; Palmer *et al.*, 2011a) have chemically modulated bacterial AHL quorum sensing in a host model to ask whether signalling affects colonization robustness in the host environment, and all of these studies have focused on pathogenic associations (Bjarnsholt and Givskov, 2007; O'Loughlin *et al.*, 2013). Pathogens represent only a small fraction of the microbes that both encode LuxIR-type systems and colonize animal or plant hosts; thus, we chose to apply a chemical approach, in combination with existing strains of *Vibrio fischeri* carrying mutations in AinS-LitR and LuxIR branches of quorum sensing, to study the role of the LuxIR signal circuit in the maintenance of stable, beneficial host–microbe associations.

The symbiosis between the marine bacterium *V. fischeri* and the squid *Euprymna scolopes* is a model system to study the initiation and maintenance of a natural, two-partner mutualism (Mandel, 2010). A monospecific and extracellular population of *V. fischeri* is maintained in a specialized host structure called the light

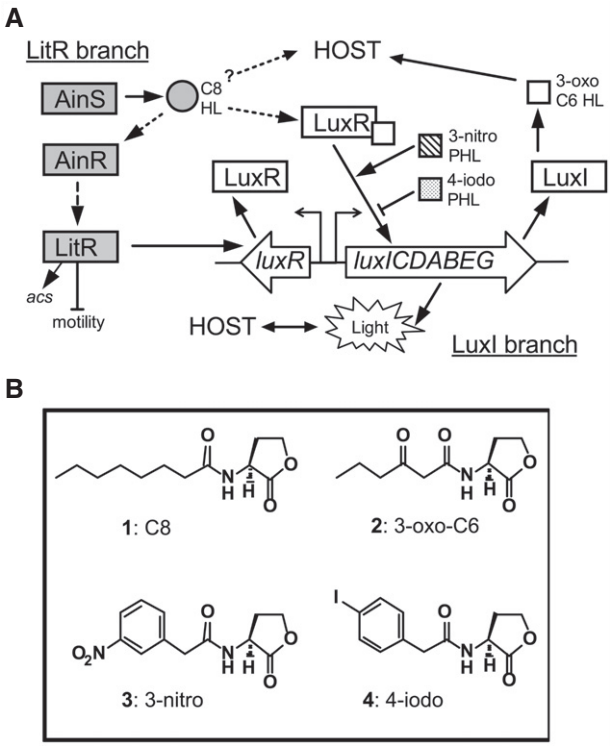

Fig. 1. The core AHL-dependent pathways of quorum signalling in *V. fischeri*.
A. AinS synthesizes C8 HL, which is released into the environment and, when at a sufficiently high concentration, diffuses back into the cell. Subsequent interaction of C8 HL with LuxR may potentiate a weak, but potentially priming, induction of the *lux* operon (*luxICDABEG*). When a threshold concentration of C8 HL is reached, C8 HL is thought to bind its cognate receptor AinR, initiating a multi-step signalling cascade leading to an increase in LitR translation (Kimbrough and Stabb, 2013). LitR further activates transcription of *luxR*, increases the amount of LuxR. When it binds 3-oxo-C6, LuxR strongly activates transcription of the *luxICDABEG* operon. Activation of *luxI* transcription increases the synthesis of 3-oxo-C6, and amplifies induction of the *lux* operon, leading to an exponential increase (autoinduction) in the synthesis of the luciferase complex and light production. 3-nitro PHL and 4-iodo PHL are structural analogues of the HL family of quorum-sensing signals, and specifically enhance or depress LuxR function respectively. The presence of native AHL molecules, C8 HL and 3-oxo-C6 HL has been shown to also alter host gene expression.
B. Structures of the natural autoinducers (1 and 2) and non-native autoinducer analogues (3 and 4) used in this study: (1) octanoyl homoserine lactone; (2) 3-oxo-hexanoyl homoserine lactone; (3) 3-nitrophenyl homoserine lactone; and (4) 4-iodophenyl homoserine lactone.

organ, where, as the name would suggest, symbionts produce light in exchange for the habitat provided by the host. Bioluminescence, and other behaviours that promote the stable association of a microbe and its host, are regulated by quorum sensing in *V. fischeri* (Stabb and Visick, 2013).

The principal quorum-sensing circuit in *V. fischeri* is composed of the AHL signal molecule *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6 HL), the signal synthase LuxI and the AHL-binding transcriptional regulatory element LuxR. The LuxIR circuit is auto-regulated; LuxR bound to an AHL signal activates transcription of *luxI*, and promotes accumulation of the LuxI-synthesized 3-oxo-C6 HL (Fig. 1A). In addition to LuxIR, *V. fischeri* encodes a second AHL-based quorum-sensing system, which is mediated by the *N*-octanoyl L-homoserine lactone (C8 HL) signal synthesized by AinS (Fig. 1A). The C8 HL signals through the histidine kinase AinR in a concentration-dependent manner (Gilson *et al*., 1995; Lupp *et al*., 2003; Kimbrough and Stabb, 2013). A third, non-AHL, quorum signal is expressed in *V. fischeri*: a furanosyl borate diester (AI-2) that is synthesized by LuxS, and likely signals through LuxP and LuxQ, similar to the homologous set of genes in *Vibrio harveyi* (Lupp and Ruby, 2004; Neiditch *et al*., 2006).

All quorum-sensing pathways in *V. fischeri* intersect at LuxR (Fig. 1A). We have previously shown that in culture, both C8 HL and AI-2 accumulation contribute to activation of *luxR* transcriptional activator LitR (Fig. 1A) (Lupp *et al*., 2003; Lupp and Ruby, 2004). C8 HL may also weakly bind to the non-cognate receptor LuxR, and contribute to an additional overlap between signalling systems (Fig. 1A) (Dunlap, 1999; Lupp *et al*., 2003). In addition to the downstream targets of LuxR regulation (Lupp and Ruby, 2005; Antunes *et al*., 2007), C8 HL controls an extensive set of genes, independent of LuxR (Lupp and Ruby, 2005; Antunes *et al*., 2007).

These convergent signal cascades culminate with the transcriptional regulation of the *luxICDABEG* operon, which encodes the light-producing luciferase enzyme complex, as well as LuxI itself. Previous studies suggest that regulation by AHL quorum sensing, mediated by AinS and LuxI, is necessary for colonization and bioluminescence of *V. fischeri* in the squid host, while the contribution of LuxS signalling is not essential for either process (Lupp and Ruby, 2004). The bioluminescence of *V. fischeri* is required to maintain a stable, and long-term partnership between host and symbiont, and possibly to signal the host (Heath-Heckman *et al*., 2013; Koch *et al*., 2013). A recently recognized role for quorum signals is as effectors of cross-kingdom communication (Rumbaugh and Kaufmann, 2012); notably, the *E. scolopes* transcriptome responds to the presence of LuxI signal 3-oxo-C6 HL (Chun *et al*., 2008).

Despite the centrality of quorum sensing in the 'conversation' between squid and vibrio, much work remains to decipher to contribution of this regulatory network and its signals to the establishment and maintenance of a stable and robust symbiosis. To bring a new perspective to studies of quorum regulation and signalling in the squid–vibrio association, we took a combined chemical and genetic approach to disentangle the signalling cascade upstream and downstream of the regulatory

element LitR, and to assess the relative contributions of each branch of the cascade to colonization of *E. scolopes*. We have identified a series of synthetic, non-native AHLs that are capable of strongly modulating luminescence (presumably *via* interaction with LuxR) in laboratory cultures of *V. fischeri* ES114, a squid light-organ isolate (Geske *et al*., 2007a,b; 2008). Among these compounds, the phenylacetanoyl homoserine lactone (PHL) derivatives were found to be the most potent as both agonists and antagonists of luminescence. Two PHLs of particular interest were 3-nitro PHL, an agonist that, at a comparable molarity, induced higher levels of luminescence than the native LuxR ligand 3-oxo-C6 HL, and 4-iodo PHL, an antagonist that strongly inhibited luminescence induction by 3-oxo-C6 HL (structures shown in Fig. 1B).

In the current study, we show that in *V. fischeri*, (i) the effects of these two PHL derivatives are specific to targets of the signal synthesized by LuxI, and do not perturb known LuxI-independent quorum signalling, and (ii) these compounds can be used, at concentrations comparable with those observed in culture studies (Geske *et al*., 2007a), to manipulate bacterial activities within the squid host. Our chemical studies highlight an intriguing and novel role for LuxIR quorum sensing during the initial stages of the symbiotic 'conversation'. Specifically, we demonstrate that the first day of colonization is a critical period in which regulation by LuxIR quorum sensing predicates the long-term stability of the symbiotic partners. These studies underscore the value of chemical methods to probe mechanisms of quorum sensing and, to our knowledge, do so for the first time in the context of a beneficial eukaryotic-prokaryotic symbiosis.

Results

Non-native analogues of 3-oxo-C6 HL do not affect AinS phenotypes

Certain native AHL autoinducers or non-native antagonists are able to signal through two AHL receptors (Schaefer *et al*., 1996; Swem *et al*., 2009; Palmer *et al*., 2011a,b). To determine whether the PHL analogues interact only with the LuxI-dependent quorum-sensing pathway, or if the molecules also modulate quorum signalling upstream of LuxI, we examined the effects of added native autoinducer and non-native PHLs on two phenotypes that are regulated by LitR and AinS, but that are independent of the LuxI branch of quorum sensing in *V. fischeri* (Fig. 1B). Using concentrations of native and non-native AHLs sufficient to modulate LuxI signalling in previous studies (Geske *et al*., 2007a), we examined motility on soft agar (repressed by LitR and AinS), and the metabolic shift known as the acetate switch (activated by LitR and AinS) (Figs 1A and 2).

We first performed motility assays to determine the effects of the native AHLs and the PHL analogues on AinS and LitR-dependent signalling. As previously reported (Lupp and Ruby, 2005), cells of an *ainS* mutant spotted onto soft-agar medium swam faster, and produced significantly larger outer rings of migration, than those of their wild-type parent, consistent with AinS- and LitR-dependent repression of motility (Fig. 2A). Previous work has demonstrated that none the molecules used in the present study alters growth of *V. fischeri* (Geske *et al*., 2007a). As anticipated, *ainS*$^-$ cells spotted onto agar containing C8 HL migrated the same distance as their similarly treated parent (Fig. 1A), although neither 3-oxo-C6 nor the PHL analogues influenced migration. The addition of 3-nitro PHL slightly increased the motility of the *litR* mutant but not the *ainS* mutant relative to wild type, while 4-iodo PHL had no effect on the migration of either mutant. The effect of 3-nitro PHL on motility was slight compared with that of C8 HL; however, it is possible that this analogue may also contribute to LitR-dependent signalling. Notably, the effect of 3-nitro PHL was only apparent in the absence of C8 HL, suggesting that the signalling interaction would not be biologically relevant in an environment where C8 HL accumulates, such as the squid light organ. This hypothesis is consistent with the finding that 3-oxo-C6 HL may also have the capacity to signal via the LitR-dependent pathway, although with much less sensitivity than 3-oxo-C6 HL-LuxR signalling (Kimbrough and Stabb, 2013).

The transcription of the acetyl-CoA synthetase gene, *acs*, is a measure of the alteration of the AinS-dependent induction of the acetate switch (Fig. 1A). Wild-type and *ainS*-mutant strains, each carrying an *acs'-lacZ*+ promoter-reporter plasmid, were grown in liquid medium supplemented with the native autoinducer or non-native analogue, and assayed for *acs* expression (Fig. 2B). Consistent with previous results (Studer *et al*., 2008), without any additions, *ainS*$^-$ *V. fischeri* had a 41% lower level of *acs* expression relative to wild-type cells. Neither the native autoinducer nor the PHL analogues significantly altered the expression of *acs* in the wild-type cultures. In contrast, the addition of C8 HL to the medium raised *acs* expression in the *ainS* mutant to 110% of wild-type levels, as expected (Studer *et al*., 2008). Further, 3-oxo-C6 HL, 3-nitro PHL and 4-iodo PHL did not significantly alter the expression by *ainS*$^-$ *V. fischeri*, indicating that 3-nitro PHL is unable to induce the AinSR system in the absence of C8 HL, and that 4-iodo PHL does not suppress this system in wild-type cells. Together with the motility data, these results indicate that, although 3-nitro PHL and 4-iodo PHL interact with the 3-oxo-C6 HL-regulated LuxIR system, they do not alter C8 HL-regulated phenotypes

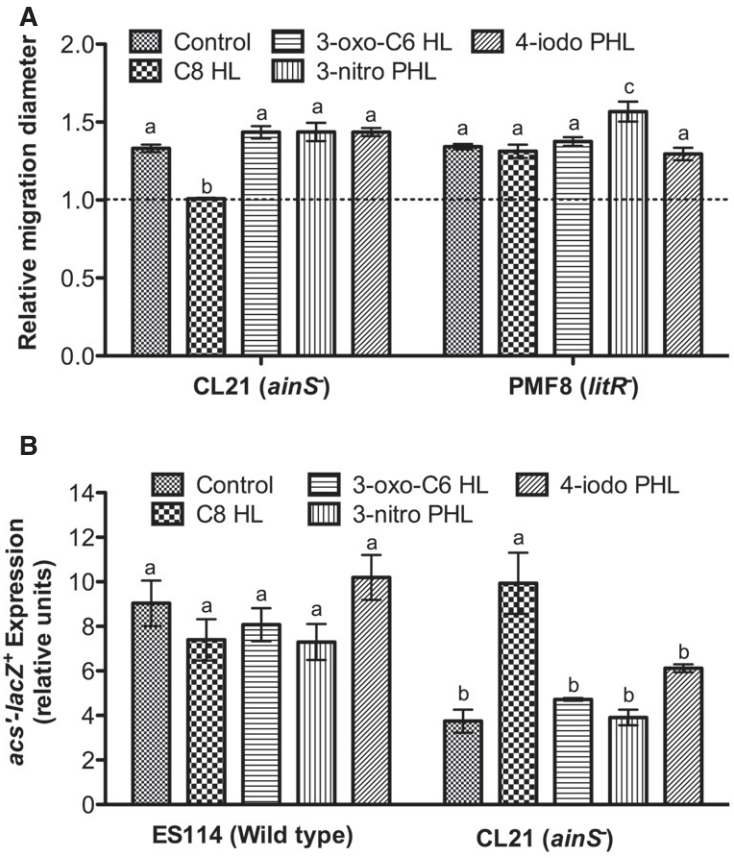

Fig. 2. AinS-dependent transcriptional and phenotypic regulation is unaffected by non-native PHL derivatives.
A. The migration of the indicated mutant strains of *ainS*$^-$ and *litR*$^-$ *V. fischeri* was measured, relative to migration of a wild-type control, after 16 h on motility agar supplemented with either a solvent control (DMSO), or 120 nM of the indicated HL or PHL analogue. Error bars represent standard error of the mean (SEM) of nine replicate plates. 'a', 'b' and 'c' indicate distinct treatment groups with statistically equivalent means; two-way analysis of variance (ANOVA) (interaction $F(4,80) = 10.1$, $P < 0.001$; HL addition $F(4,80) = 20.8$, $P < 0.0001$; strain $F(1,80) = 4.0$, $P = 0.049$), with post-hoc Bonferroni *t*-tests.
B. The level of *acs* expression in wild-type and *ainS*$^-$ strains of *V. fischeri* containing the *acs'-lacZ*$^+$ reporter plasmid were determined after 4 h of growth in SWT supplemented with 40 nM of the indicated AHL or PHL analogue. Error bars represent SEM of four biological replicates. Significance was determined by two-way analysis of variance (ANOVA) (interaction $F(4,20) = 7.2$, $P = 0.0009$; strain $F(1,20) = 28.7$, $P < 0.0001$; addition $F(4,20) = 5.3$, $P = 0.0045$), with post-hoc Bonferroni comparison testing: 'a' and 'b' indicate distinct treatment groups with statistically equivalent means.

through LitR, and therefore do not target this branch of quorum sensing in *V. fischeri* in an environment with active C8 HL signalling.

Native and non-native LuxR-binding molecules can signal during symbiosis

To determine whether experimentally added autoinducer signals affect *V. fischeri* during colonization of its natural host, we first examined whether the presence of 4-iodo PHL, an antagonist of 3-oxo-C6 HL in culture (Geske *et al*., 2007a), was also effective in *V. fischeri* within the host. Specifically, we asked whether 4-iodo PHL could antagonize LuxIR quorum sensing in the host, and reduce the level of wild-type bioluminescence to that of mutants deficient in LuxIR signalling. Wild-type colonized animals maintained in seawater containing 50 or 100 μM 4-iodo PHL had 20- to 40-fold lower levels of luminescence at 48 h of colonization than in seawater without added antagonist (Fig. 3A). As little as 5 μM, 4-iodo PHL was sufficient to reduce the bioluminescence of *V. fischeri* cells to the level of the *luxI* mutant, which attains approximately 5% of the bioluminescence of wild-type cells in symbiosis, while a concentration of 0.5 μM had no significant effect on symbiotic bioluminescence (Fig. 3A). Notably, the amount of 4-iodo PHL observed to reduce bioluminescence is within the range of the concentration for 50% inhibition (IC_{50}) for this molecule obtained in culture studies (IC_{50} = 0.9 mM) (Geske *et al*., 2007a), and suggests that the delivery of the analogues to the symbiont population is not greatly hindered by host tissues.

We next measured the contribution of native and non-native LuxR agonists to the induction of bioluminescence. As expected (Visick *et al*., 2000), in the absence of added signal, the bioluminescence of animals colonized by the *luxI* mutant was lower than that of wild-type colonized animals (Fig. 3B and C). In contrast, addition of as little as 0.5 mM of either 3-oxo-C6 HL (Fig. 3B) or 3-nitro PHL (Fig. 3C) to animals colonized by *luxI*$^-$ *V. fischeri* restored induction of bioluminescence. Higher concentrations of the experimentally added signals did not further increase the bioluminescence of either wild-type *V. fischeri*, or the *luxI* mutant, and we noted no significant difference in the maximum level of bioluminescence achieved by the superactivator 3-nitro PHL when compared with the native LuxR agonist, 3-oxo-C6 (concentration for 50% activation, EC_{50} values = 0.3 and 3.0 mM, respectively, in *V. fischeri* culture; Geske *et al*., 2007a). It is still possible that the concentration of exogenously added signal experienced by the symbionts inside the squid's light organ is lower

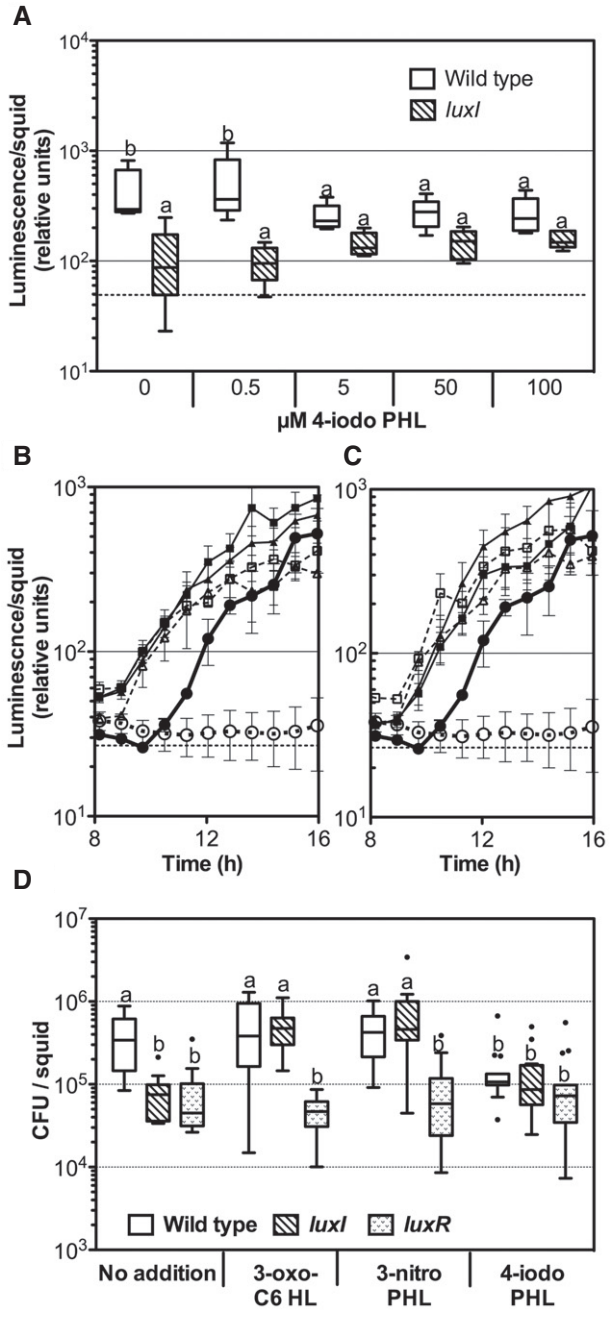

Fig. 3. Contribution of native and non-native HL analogues to symbiotic colonization and bioluminescence at 48 h post-inoculation.
A. Bioluminescence of wild-type (open boxes) was compared with *luxI*$^-$ *V. fischeri* (diagonal boxes) in the presence of different concentrations of 4-iodo PHL. Significant differences in mean bioluminescence were determined by the Kruskal–Wallis test ($H = 36.2$, 10 d.f., $P < 0.0001$), with post-hoc Dunn's multiple comparisons ('a' and 'b' indicate distinct groups with equivalent means; $P < 0.05$). Inner fences determined by Tukey's method, dashed line indicates background level of luminescence. Induction of bioluminescence of wild type (closed symbols) or *luxI* (open symbols) was monitored in the presence of
B. no added HL (circles), 0.5 μM C6 HL (triangles), or 5 μM C6 HL (squares), or
C. no added HL (circles), 0.5 μM 3-nitro PHL (triangles), or 5 μM 3-nitro PHL (squares). Bars represent standard error of the mean (SEM) of $n = 5$; dashed line indicates level of detectable luminescence.
D. Animal colonization at 48 h was compared between inoculations with wild-type (open boxes), *luxI*$^-$ (diagonal boxes) or *luxR*$^-$ (dotted boxes) *V. fischeri* in the presence of 5 μM added HL, as indicated. Significant differences in mean colony-forming units (CFU) per animal were determined by the Kruskal–Wallis test ($H = 172.4$, 15 d.f., $P < 0.0001$), with post-hoc Dunn's multiple comparisons ('a' and 'b' indicate distinct groups with equivalent means; $P < 0.05$). Inner fences determined by Tukey's method; dots represent outliers.

than that present in the surrounding seawater because of diffusion limitation; however, addition of either 3-oxo-C6 HL (Fig. 3B) or 3-nitro PHL (Fig. 3C) resulted in induction of luminescence in wild-type colonized animals 1 h earlier than it occurred without the added compounds, suggesting that both compounds swiftly and effectively diffuse into the light organ, reaching concentrations that result in induction of LuxIR activity.

LuxIR signalling is required for *V. fischeri* to maintain a colonization of the light organ; thus, we also examined the effect of exogenously added autoinducers on colonization levels (i.e. colony-forming units (CFU) per squid) of LuxIR pathway mutants. Addition of 5 μM of the LuxR antagonist 4-iodo PHL to wild type *V. fischeri* diminished the host's maintenance of a light organ colonization to a level typical of *luxI*$^-$ *V. fischeri* (Fig. 3D). However, 4-iodo PHL had no effect on the colonization of the *luxI* mutant, supporting the conclusion that the molecule suppresses colonization by wild-type cells solely through its inhibition of the LuxIR pathway. Colonization levels of the *luxI* mutant in the squid light organ were fully restored to those of wild-type *V. fischeri* when either 3-oxo-C6 HL or 3-nitro PHL were added (Fig. 3D). Additions of 5 μM 3-oxo-C6, 3-nitro PHL or 4-iodo PHL (Fig. 3D) had no impact on the colonization defect of the *luxR* mutant, indicating that the distinct effects of both these autoinducer analogues are dependent on a functioning LuxIR signal pathway. Taken together, these results show that exogenously added autoinducers, both the native signal and the non-native agonist, induce expected LuxIR-system responses, even when the bacteria are within host tissues.

LuxR ligands do not perturb AinS-dependent symbiosis factors

To assess the effect of exogenous autoinducers on AinS-dependent quorum sensing during squid colonization by *V. fischeri*, animals colonized by wild-type, *ainS*$^-$ or *litR*$^-$ strains were exposed to either the native autoinducers or the non-native analogues. In the absence of added

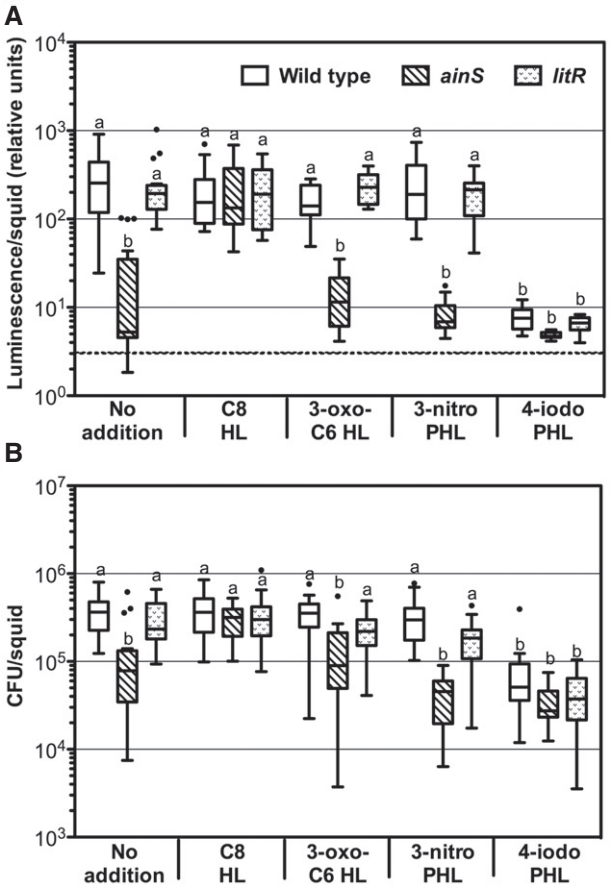

Fig. 4. AinS- and LitR-dependent colonization dynamics are not perturbed by analogues of 3-oxo-C6 homoserine lactone. Squid colonized by wild-type (white), *ainS*⁻ (diagonal) or *litR*⁻ (dotted) *V. fischeri* were treated with either a DMSO solvent control (no addition), or 5 μM additions of C8 HL, 3-oxo-C6 HL, 3-nitro PHL or 4-iodo PHL as indicated.
A. Differences in bioluminescence among treatments and strains at 48 h were assessed by the Kruskal–Wallis test ($H = 78.8$, 15 d.f., $P < 0.0001$), with post-hoc Dunn's multiple comparisons ('a' and 'b' indicate distinct groups with equivalent means, $P < 0.05$); dashed line indicates background level of luminescence.
B. Differences in colonization levels at 48 h were assessed by the Kruskal–Wallis test ($H = 149.7$, 15 d.f., $P < 0.0001$) with post-hoc Dunn's multiple comparisons ('a' and 'b' indicate distinct groups with equivalent means, $P < 0.05$). Inner fences determined by the Tukey method; black dots indicate outliers; CFU, colony-forming units.

compounds, animals colonized by the *ainS* mutant had both a lower level of luminescence (Fig. 4A) and a lower number of CFU/squid (Fig. 4B) when compared with wild-type colonized animals. Luminescence and colonization by *litR*⁻ *V. fischeri* were indistinguishable from wild-type *V. fischeri* in the absence of added compounds (Fig. 4A and B). The addition of C8 HL, but not 3-oxo-C6 HL, restored the luminescence and CFU/squid of animals colonized by the *ainS* mutant to wild-type levels, demonstrating that the mutant's defect in symbiosis is not caused solely by a repression of signal synthesis by LuxI. The addition of the antagonist, 4-iodo PHL, decreased the luminescence and colonization levels of all strains, further indicating that the LuxIR system is active, and subject to inhibition, during colonization of squid by *ainS*-deficient symbionts.

Because there are regulatory targets of LuxIR quorum sensing other than the bioluminescence gene cluster *luxCDABEG* (Antunes *et al.*, 2007), we reasoned that if a target of LuxIR quorum sensing apart from bioluminescence contributed to host colonization, the symbiotic population would be sensitive to the addition of LuxR antagonist 4-iodo PHL, even in the absence of the *luxCDABEG* genes. Accordingly, we observed that the level of the symbiotic population of a Δ*luxCDABEG* mutant (Bose *et al.*, 2008), which lacks the genes that encode the luciferase complex, was significantly lower than that of wild-type *V. fischeri* at 48 h post-colonization (Fig. S1). While the population level of Δ*luxCDABEG* cells was not altered by 5 μM 4-iodo PHL, the addition of 4-iodo PHL to a wild-type colonization did not completely decrease the level of symbiotic bioluminescence to that of Δ*luxCDABEG* (data not shown), and this small amount of residual bioluminescence correlated with a slightly larger population (Fig. S1). Together, these data suggest that bioluminescence is the key LuxIR-regulated factor that contributes to initial light-organ colonization, and that even a low level of light production during symbiosis can contribute to the maintenance of a normal symbiont population level.

An initial, yet transitory, appearance of bioluminescence is sufficient to result in robust and persistent colonization

LuxIR induction of bioluminescence is required for *V. fischeri* to maintain colonization of the light organ, but the population levels of Δ*luxCDABEG*, *luxI* and *luxR* mutants only begin to decrease after the first 24 h of symbiosis (Visick *et al.*, 2000; Bose *et al.*, 2008). Surprisingly, we found that chemically inhibiting LuxIR activation of bioluminescence by the addition of 5 μM 4-iodo PHL after 24 h of light-organ colonization did not significantly alter colonization of *V. fischeri* at 48 h (Fig. 5), even though addition of the antagonist was sufficient to lower symbiotic bioluminescence to levels comparable with the colonization-deficient *luxI*⁻ strain. More unexpected was the observation that, after 24 h or colonization, even doubling the length of the symbiont's exposure to 4-iodo PHL had no significant impact on colonization of the light organ (Fig. 5). Collectively, these results suggest that induction of bioluminescence by LuxIR within the first 24 h of symbiosis is sufficient to maintain robust symbiont colonization more than a day later.

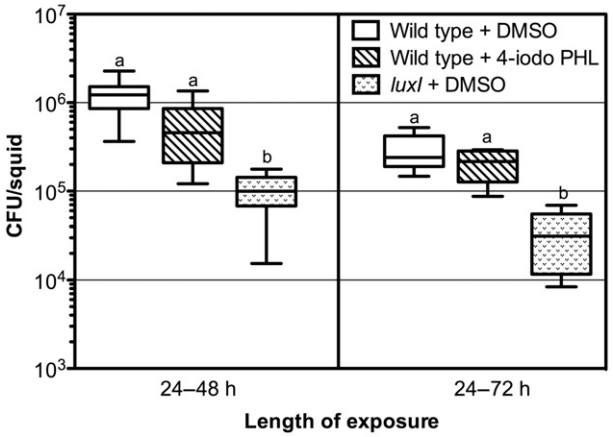

Fig. 5. Squid colonized with wild-type *V. fischeri* for 24 were either left untreated (open boxes), or treated with 4-iodo PHL (diagonal boxes) for an additional 24 h (end-point = 48 h), or 48 h (end-point = 72 h) prior to enumeration of colony-forming units (CFU). Populations of squid were also colonized by *luxI*$^-$ in the presence of a DMSO solvent control (dotted boxes) as a comparison for 4-iodo PHL inhibitory activity. The mean CFU value was compared with that of the control wild-type populations by Kruskal–Wallis test ($H = 47.9$, 6 d.f., $P < 0.0001$), with post-hoc Dunn's multiple comparisons ('a' and 'b' indicate distinct groups with equivalent means, $P < 0.05$).

To better define the window in which LuxIR-regulated bioluminescence is critical for persistent colonization of the host, we perturbed the LuxIR circuit by addition of PHL analogues in the symbiosis after 18 h of infection. We reasoned that by this point in the symbiosis: (i) the symbiont has reached its peak population in the light organ (Ruby, 1996), and (ii) the host has initiated irreversible, symbiont-dependent, morphogenic programmes (Nyholm and McFall-Ngai, 2004). We first modulated the loss or gain of bioluminescence at 18 h either by adding 4-iodo PHL to squid colonized by wild-type *V. fischeri*, or by adding 3-nitro PHL to squid colonized by *luxI*$^-$ *V. fischeri* from 18 to 48 h post-colonization. The rapid loss of animal bioluminescence upon addition of 4-iodo PHL at 18 h (Fig. 6A), which occurred within a few hours of exposure to the antagonist (Fig. S2), was sufficient to decrease the population of wild-type *V. fischeri* to levels comparable with *luxI* by 48 h of colonization (Fig. 6B). Additionally, the rapid gain of bioluminescence achieved by exposing squid colonized by the *luxI* mutant to 3-nitro PHL after 18 h of infection (Fig. 6B) did not lead to a significant increase in the colonization level of *luxI*$^-$ *V. fischeri* at 48 h (Fig. 6B). Taken together, these data suggest that continual activation of bioluminescence by LuxR, throughout the first day of colonization, rather than the induction of bioluminescence at a specific stage during the initiation of symbiosis (Nyholm and McFall-Ngai, 2004), is required to maintain a robust population over subsequent days.

If the level of bioluminescence, rather than simply its presence, is the critical determinant of colonization stability, then we reasoned that a gradual decrease in LuxR activation of bioluminescence after 18 h should not impair colonization as severely as the complete loss of bioluminescence at that juncture. To test this hypothesis, we

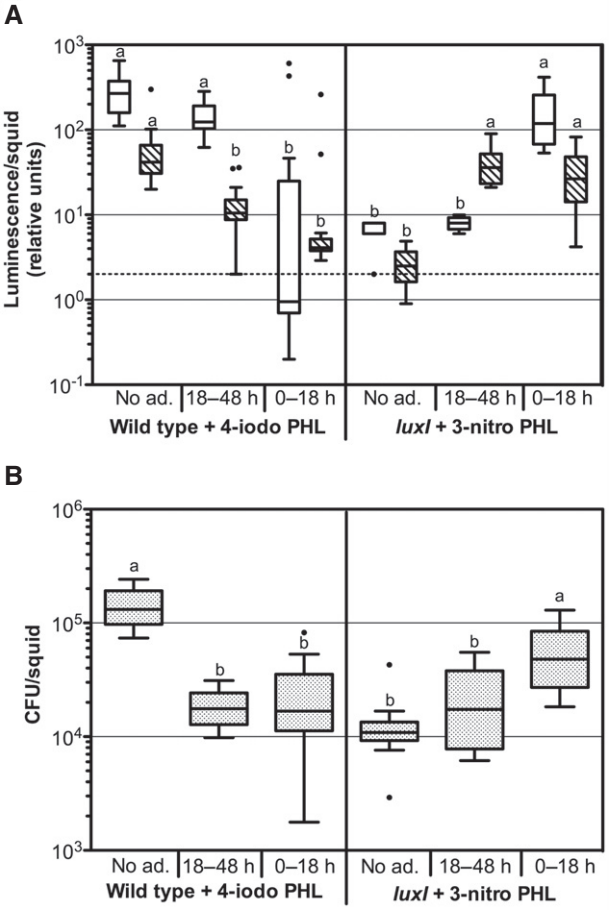

Fig. 6. Induction of bioluminescence by LuxIR quorum sensing is required for establishment of a robust symbiont population. The bioluminescence of an established symbiont population was either inhibited or induced by exogenous exposure to 5 μM of an HL-analogue for a period of time (exposure indicated as a range of hours post-colonization: 0–18 h or 18–48 h; No ad. = no analogue addition).
A. Animal bioluminescence was measured at 18 h (open boxes) and 36 h (diagonal boxes) post-inoculation for each treatment group. Differences in mean luminescence (compared with wild type without additions) were determined by the Kruskal–Wallis test ($H = 147.3$, 12 d.f., $P < 0.0001$) with post-hoc Dunn's multiple comparisons ('a' and 'b' indicate distinct groups with equivalent means); dashed line indicates threshold of bioluminescence detection.
B. Mean CFU at 48 h post-inoculation was compared with the wild type without additions by the Kruskal–Wallis test ($H = 41.3$, 6 d.f., $P < 0.0001$), with post-hoc Dunn's multiple comparisons ('a', 'b' and 'c' indicate distinct groups with equivalent means, $P < 0.05$). Inner fences determined by the Tukey method; black dots indicate outliers; CFU, colony-forming units.

exposed squid colonized by wild-type *V. fischeri* to 4-iodo PHL (to diminish luminescence), or *luxI*$^{-}$ *V. fischeri* to 3-nitro PHL (to enhance luminescence) from 0 to 18 h of colonization. The small change in the mean luminescence levels of the animals is consistent with the retention of some of the molecule by the animals (Fig. 6A). The slight increase of wild-type bioluminescence observed after removal of 4-iodo PHL at 18 h was not sufficient to support symbiotic colonization at 48 h, consistent with the defect in colonization observed in squid colonized by non-bioluminescent symbionts from 0 to 18 h (Fig. 6B). In contrast, the gradual decrease in bioluminescence of the *luxI* mutant following 18 h exposure to 3-nitro PHL only slightly impaired symbiont colonization at 48 h, compared with the colonization level of wild-type *V. fischeri* in which bioluminescence had been severely impaired by addition of 4-iodo PHL from 18 to 48 h (Fig. 6B). These results suggest that the continued maintenance of a stable symbiont population requires that the symbionts achieve a threshold level of bioluminescence during the first day of colonization.

Discussion

Synthetic small-molecule probes can help illuminate biological phenomena by providing both temporal and spatial levels of control to experimental manipulations and, thereby, complement traditional biochemical and genetic approaches (O' Connor *et al*., 2011). These tools are particularly powerful for the study of both intercellular signalling and developmental processes, where the timing and sequence of extracellular trigger molecules play crucial roles. As highlighted above, the design and application of such chemical probes to interrogate bacterial quorum sensing has recently become a target of intense study (Rasmussen and Givskov, 2006; Amara *et al*., 2011; Galloway *et al*., 2011; Praneenararat *et al*., 2012). Much of the practical interest in quorum-signal manipulation comes from its potential to create a new class of anti-pathogenic agents designed, for example, to inhibit bacterial virulence (Njoroge and Sperandio, 2009; Lowery *et al*., 2010). However, the use of such therapies to modulate bacterial pathogens within animal tissues requires a much clearer understanding of the response of the co-occurring normal microbiota to any unintended collateral effects of the treatment on their quorum-sensing processes.

The remarkable specificity that the light-organ environment shows for *V. fischeri* makes the squid–vibrio symbiosis ideal for the study of the chemical dialog that promotes a long-term and stable association. In the current study, we report for the first time the use of non-native, synthetic AHLs (3-nitro and 4-iodo PHLs) to probe this symbiosis. As in the squid–vibrio system (Lupp and Ruby, 2005; Antunes *et al*., 2007), the legume–rhizobium mutualism also promotes the establishment of a stable colonization by activating genes required for the earliest stages of nodulation in the host (González and Marketon, 2003). In contrast, quorum sensing by the pathogens *Vibrio cholerae* (Liu *et al*., 2008) and *Pectobacterium carotovora* (Palmer *et al*., 2011a) *represses* production of the bacterium's virulence factors, presumably to evade host defences and/or delay their deployment until the population is dense enough to sustain or transmit an infection (von Bodman *et al*., 2003). Thus, in both mutualism and pathogenesis, the timing of signalling during host colonization is the key, and addition of chemical signal analogues can help probe this temporal pattern. In addition, it is tempting to speculate that differences in the 'wiring' of quorum-sensing systems among pathogens and mutualists of the same genus, such as *V. cholerae* and *V. fischeri*, contribute in some way to the dichotomy of host responses.

Specific and sensitive chemical modulation of LuxIR quorum sensing

To interpret experiments designed to chemically manipulate quorum sensing, it is necessary for any small-molecule analogues to be specific. *V. fischeri*, like many Gram-negative microbes that encode LuxIR quorum-signalling circuits, encodes an additional AHL quorum signal, synthesized by AinS (Gilson *et al*., 1995; Stabb *et al*., 2007). Although the putative receptor, AinR, is not structurally related to LuxR, both may bind acyl-homoserine lactone signals, including 3-oxo-C6 HL, and C8 HL (Miyashiro and Ruby, 2012; Kimbrough and Stabb, 2013). Furthermore, LuxR has previously been shown to react to the AinS signal, C8 HL and AinR to the LuxR signal 3-oxo-C6 HL, although neither receptor is as sensitive to the non-cognate signal (Fig. 1A) (Lupp *et al*., 2003; Kimbrough and Stabb, 2013). Despite this indication of cross-talk between the natural AHLs of *V. fischeri*, our phenotypic, transcriptional and symbiotic measurements reported herein indicate that, in the context of functional C8 signalling, the two non-native PHLs do not influence any *ainS*-dependent phenotypes. In the context of the symbiosis, C8 HL signalling does not function solely as a mechanism by which to induce bioluminescence at low cell density; in fact, we noted that activation of LuxIR by the exogenous addition of a LuxR agonist was not sufficient to restore normal colonization of the host by an *ainS* mutant. This finding supports the hypothesis that LuxIR-independent targets of AinSR regulation, such as motility and acetyl-CoA synthase (Lupp and Ruby, 2005; Studer *et al*., 2008), are required for persistent colonization of the light organ. Perhaps the key to quorum signalling in the context of symbiosis lies in the relative

abundance of each AHL signal and the regulation surrounding these levels.

We also considered the characteristics of LuxIR-mediated quorum sensing by the PHL analogues during light-organ colonization. Both PHL molecules were able to modulate LuxIR signalling at concentrations as low as those previously reported in laboratory-culture experiments (Geske *et al*., 2007a). In addition to LuxR activation being highly sensitive to the PHLs, the transient addition of the analogues had a long-lived effects; that is, the symbionts of animals that had been removed from seawater containing PHL continued to respond for up to 1 day as though the analogue were present. This observation is consistent with the light-organ crypts being a diffusion-limited environment, and suggests that the ability to experimentally reverse the action of these small chemical probes will be limited in the context of this, and perhaps other, animal models.

Interestingly, while the analogues retained their efficacy in the symbiotic environment, in the squid, we did not observe that the 3-nitro PHL agonist had a higher specific activity of bioluminescence induction than the natural agonist, as had been found in culture (Geske *et al*., 2007b). Because the host's control of bioluminescence in the light organ is likely to rely on several physiological tissue conditions, such as oxygen availability (Miyashiro and Ruby, 2012), it is possible that these environmental factors, rather than LuxIR signalling, determine the maximum level of bioluminescence produced by *V. fischeri* cells in the light organ.

Bioluminescence leaves a persistent 'first impression'

The chemical manipulation of LuxIR quorum sensing described here offers new insight into the process by which a robust and stable mutualism is established between *V. fischeri* and *E. scolopes*. We show that LuxIR quorum signalling enhances the transition of symbiotic *V. fischeri* from an initial colonization of the host to a robust, and long-term association, and that the presence of animal bioluminescence during the first 24 h of colonization is predictive of symbiont population dynamics over subsequent days of the symbiosis. Our results define a preliminary framework on which to map existing and future studies of microbial colonization factors and, thereby, suggest principles of host selection and maintenance of a bioluminescent symbiont during initiation of the squid–vibrio mutualism.

The contribution of AHL molecules to the chemical dialogue between host and symbiont cannot be ignored (Chun *et al*., 2008) (Fig. 1A); however, our data suggest that direct chemical signalling does not itself contribute to the host's control of the number of symbionts in the light organ. Rather, we propose that the host's perception of bioluminescence, or of a product of the bioluminescence reaction itself, is the signal or cue that governs maintenance of symbiont population levels. The centrality of light as a cue for maintenance of a stable symbiosis is consistent with the structural and physiological attributes of the light organ: symbiont bioluminescence is perceived by the tissues of this specialized structure (Tong *et al*., 2009), and these tissues also have the capacity to entrain physiological rhythms based on this stimulus (Heath-Heckman *et al*., 2013).

Our data suggest that, below a minimum threshold of bioluminescence, the benefit of bioluminescence to the symbiont is proportional to the amount of light produced. For example, we show that, even in the absence of LuxIR signalling, there is a small amount of light produced by the basal level of luciferase, and this activity may enhance the fitness of *luxI* and *luxR* mutants, relative to a completely 'dark' strain missing the genes encoding luciferase itself. Further, our temporal manipulation of light production during the first 24 h after symbiotic infection indicates the critical contribution of light to the persistent colonization of the light organ. However, our data also demonstrate that even a slowly diminishing bioluminescence output, if it does not fall below a minimum threshold, is sufficient for maintenance of the symbiont population by the host. Finally, we show for the first time that bioluminescence produced by *V. fischeri* within the first day of colonization appears to be a critical determinant of symbiotic population dynamics over the longer term. It will be interesting to elucidate the mechanism(s) by which this initial bioluminescent 'conversation' between host and symbiont is predictive of the long-term stability of the partnership.

Conclusions

Regulation of microbial colonization factors by quorum signalling is rarely studied within the host itself. Nevertheless, it is perhaps from this vantage that the contribution of quorum sensing to bacterial population dynamics may be best understood. In this study, we demonstrate that native and non-native HL analogues can be used to modulate LuxIR quorum sensing without perturbing either (i) the AinS-LitR quorum-signalling circuitry of *V. fischeri*, or (ii) non-LuxIR-dependent factors required for symbiosis with *E. scolopes*. In addition to these observations, our chemical approach for perturbing quorum-sensing circuitry suggests a novel role for LuxIR regulation of bioluminescence not revealed by previous genetic approaches. That is, we show that the LuxIR quorum-sensing circuit, from the first hours through the first day of colonization, is required to establish the stability of the mutualism, and that the success of this initial signalling has long-term consequences for the maintenance of the symbiont population

by the host. Using longitudinal studies that are becoming feasible in the squid–vibrio model (Koch *et al*., 2013), future work will determine how long the impact of the first LuxIR-mediated bioluminescent 'conversation' is reflected in the dynamics of the symbiont population.

Experimental procedures

Bacterial strains and growth conditions

Strains and plasmids used in this work are listed in Table S1. *V. fischeri* strains are derivatives of ES114 (Boettcher and Ruby, 1990), and were grown at 28°C in either a seawater-tryptone medium (SWT) (Boettcher and Ruby, 1990) or Luria-Bertani salt medium (LBS) (Graf *et al*., 1994). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook *et al*., 1989). Media were solidified with 1.5% agar as needed. When appropriate, antibiotics were added to LB and LBS media at the following concentrations: chloramphenicol, 5 μg/ml for *V. fischeri* and 25 μg/ml for *E. coli*; erythromycin, 5 μg/ml for *V. fischeri*; and kanamycin 100 μg/ml for *V. fischeri*. Medium reagents were purchased from ThermoFisher Scientific (San Diego, CA, USA).

β-galactosidase assay

The β-galactosidase activity assays were preformed as described previously (Studer *et al*., 2008). Briefly, strains were cultured in SWT, the optical density (OD) at 600 nm was measured, and β-galactosidase activity was determined after 4 h, using a microtiter-dish method modified from Slauch and Silhavy (1991). Cell pellets were frozen at −20°C before resuspension, and the sodium dodecyl sulfate/chloroform step was omitted. The A_{420} values of the microtiter wells were read every 30 s for 1 h using a GENiosPro 96-well plate reader (TECAN, Research Triangle Park, NC, USA). Three replicates of two dilutions were tested for each cell pellet. The relative units of β-galactosidase activity were calculated using the formula: rate [Vmax]/[(OD) × volume (ml)].

Motility assay

The swimming behaviour of *V. fischeri* was determined by growing strains to approximately 1.0 OD in SWT with 0.25% dimethysulfoxide (DMSO) containing the appropriate signal-molecule additions. The cells were collected by centrifugation of 1 ml of the culture, the cell pellet washed with SWT, and resuspended to 0.6 OD units in SWT containing the additions. Three microlitres of a suspension of each strain was spotted onto the surface of a 0.25% agar plate containing defined seawater minimal medium base (Lupp and Ruby, 2005) supplemented with 0.3% casamino acids and 0.25% DMSO containing the appropriate signal molecules. The ring diameter was determined at 16 h as a measure of the relative rate of bacterial migration.

Chemical synthesis

Procedures for synthesizing 3-oxo-C6 HL, C8 HL, 3-nitro PHL and 4-iodo PHL have been previously described (Geske *et al*., 2007b). All compounds were suspended as stock solutions in DMSO, and were stored sealed in the freezer when not in use. In biological experiments, DMSO was added to a final concentration of 0.25% in all conditions, including those with no added compound.

Squid colonization assays

For animal colonization experiments, newly hatched squid were placed in filter-sterilized seawater (FSW) (Instant Ocean, Aquarium Systems, Mentor, OH, USA) containing the appropriate V. fisch*eri* strain at approximately 3000 CFU/ml; a control group of animals was placed in uninoculated FSW, supplemented with 0.25% DMSO containing either the natural AHL or non-native analogue, or no added compound, as indicated. All animals were transferred to fresh, uninoculated FSW at 3 h, and again at approximately 16 h, after inoculation. There was no evidence that additions of up to 100 μM of the AHLs or analogues had any adverse effects on the animals. At 48 or 72 h post-inoculation, individual animal bioluminescence was determined using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), and the animals were sacrificed to determine the CFU/squid. To monitor animal bioluminescence over time, measurements were acquired with Light a Packard Tri-Carb 2100TR scintillation counter (Packard Instruments, Meriden, CT, USA) modified to operate as an automated photometer. In all experiments, uninoculated animals had no detectible bioluminescence or CFUs.

Statistics

Statistical analysis was performed using the GraphPad Prism software package (Version 5.0, GraphPad Prism, San Diego, CA, USA). The experimental data sets were assessed for normal distribution using the D'Agostino and Pearson omnibus test (D'agostino *et al*., 1990). Data that fit a normal distribution were tested for significant variation among experimental groups by one- or two-factor analysis of variance, with post-hoc Bonferroni T-testing. Data that failed to fit a normal distribution were assessed by the Kruskal–Wallis test with Post-Hoc Dunn's multiple comparisons to identify groups of statistically equivalent means.

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Note in Proof

In a recent article (O'Loughlin *et al*., 2013), a non-native AHL was used to show that appropriately tuning, rather than completely inhibiting, quorum signaling was a key factor in *P. aeruginosa* colonization.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. LuxIR-dependent changes in symbiont population density require the bioluminescence gene cluster. Animals were colonized with wild-type or Δ*luxCDABEG V. fischeri* in the presence of a DMSO-only solvent control (open boxes), or exogenous HL 4-iodo PHL (diagonal boxes). Bars represent Tukey's inner fences, $n = 24$ squid. Significance determined by the Kruskal–Wallis test ($H = 67.5$, 4 d.f., $P < 0.0001$) with post-hoc Dunn's multiple comparisons ('a', 'b' and 'c' indicate distinct groups of equivalent means, $P < 0.05$). CFU: colony-forming units.

Fig. S2. Addition of 4-iodo PHL to squid colonized by wild-type *V. fischeri* rapidly decreases symbiotic bioluminescence. Animals were colonized by wild type (black lines), or the *luxI* mutant (grey lines), for 24 h, prior to addition of 50 μM 4-iodo PHL (dashed lines), or a DMSO-only solvent control (solid lines). Bars represent standard error of the mean, $n = 5$.

Table S1. Strains and plasmids used in this study.