## What the regulation of bacterial bioluminescence tells us about this and other bacterial group behaviours

# Who turned on the lights?

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Luminescence produced by organisms, or 'bioluminescence', holds a distinct fascination for humankind, and the study of bacterial bioluminescence has a long history in the field of microbiology. Advances in our understanding of bacterial bioluminescence have in many ways paralleled advances in the field as a whole. Intriguingly, studies of bioluminescent bacteria led to a seminal discovery in bacterial gene regulation and behaviour, because for bacteria, bioluminescence is a group activity. Bioluminescent bacteria communicate using pheromones, and as a result the regulatory decision to induce bioluminescence is only made if a group of bacteria has achieved a dense enough population to allow the build-up of pheromone. More recently, it has become clear that there are complex regulatory circuits governing not only luminescence, but also pheromone signalling itself. These additional layers of regulation pose new questions such as what are bacteria really saying to each other? Understanding regulation may also help answer ancient questions including, what use is luminescence?

Bioluminescence is widespread in nature, and observations of this phenomenon date back as far as recorded history[1,2]. In the 1700s, it was suggested that 'animacules' produce light, and as microbe hunters of the 1800s sought to isolate and cultivate specific bacteria that were invisible to the eye, yet responsible for outwardly apparent phenomena, several species of bioluminescent bacteria were discovered and described. Finding them was not difficult, and in undergraduate microbiology laboratories today students experience the same thrill of finding glowing colonies using similarly simple methods. As a rule of thumb, marine water typically contains approximately one bioluminescent colony-forming unit per millilitre if plated on to a rich salty medium. Landlocked instructors tolerant of foul odours may have students purchase and incubate marine seafood, often resulting in bioluminescent bacterial growth. Once such bioluminescent bacteria have been isolated, students can easily inoculate them on to fresh plates to generate a glowing growth (Figure 1). Such exercises retrace the steps of Heller, Pfluger, Beijerinck, Fischer and other scientists who demonstrated and described the microbial genesis of bioluminescence.

Of course, not all bioluminescence is microbial. Fireflies, for example, generate their own light. However, at least in certain respects, bacteria are unrivalled as experimental systems, and during the 20th Century scientists increasingly exploited them as models for many basic biological phenomena. Consequently, a great deal is known about bacterial bioluminescence.

## The bacteria that generate bioluminescence

Although bioluminescence is found in diverse organisms, the prokaryotes known to produce light fall into a relatively narrow phylogenetic slice of the bacteria[3]. All are Gram-negative, encompassed by three families within the gamma proteobacteria and most live in marine environments. Although bioluminescence seen in breaking waves is typically attributable to dinoflagellates, huge swathes of glowing ocean called 'milky seas' are thought to be due to bioluminescent bacteria associated with microalgal blooms. Non-marine exceptions include *Photorhabdus* species, which are symbionts of terrestrial entomopathogenic nematodes and cause the cadavers of the nematode's victims to glow. Reports of glowing wounds in humans, which were apparently fairly common during the American civil war, are often attributed to opportunistic *Photorhabdus* growth. Another exception is the marine bacterium *Vibrio cholerae*, which ventures into brackish and even fresh water.

***Key words:*** *bacterial pheromone signalling, bioluminescence,* Euprymna scolopes, *Hawaiian bobtail squid,* Vibrio fischeri, Vibrio harveyi

**Abbreviations:** 3OC6-HSL, *N*-3-oxo-hexanoyl homoserine lactone; ROS, reactive oxygen species.

Although some strains of *V. cholerae* cause outbreaks of cholera, several non-clinical isolates produce luminescence.

Although cells capable of bioluminescence are found free-living, evidence suggests that most, if not all, the bioluminescent bacteria are equipped to interact with host organisms. They can be found on and inside animals, in specialized one-species symbioses and in gut communities, in symbiotic mutualisms and in pathogenic infections. Bioluminescent bacteria typically induce bioluminescence when associated with a living or dead host, and not as a member of the dilute marine community.

Two marine bacteria have been workhorses for the study of bioluminescence and its regulation; *Vibrio harveyi* and *Vibrio fischeri*. The latter, named for the pioneering bioluminescence researcher Bernhard Fischer, is often found in specific symbioses, where a host grows it in a specialized light organ. The symbiosis between *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes*, can be initiated and effectively studied in the laboratory[4], making this bacterium an excellent model for researchers wishing to examine bioluminescent bacteria in an ecologically relevant context. Although studies of this symbiosis have gained momentum over the last two decades, they are built on a foundation of research into the physiology, biochemistry, genetics and regulation of bioluminescence dating to the early 20th Century[5].

## Making light and paying for it

Bioluminescence has evolved independently several times, but bioluminescence in bacteria is consistently underpinned by a conserved set of Lux proteins (Figure 2). *V. fischeri* and other bacteria produce light using a luciferase enzyme composed of LuxA and LuxB. Luciferase converts $FMNH_2$, $O_2$ and an aliphatic aldehyde (RCHO) to FMN, water and the corresponding aliphatic acid, giving off a photon of light in the process [6]. LuxD generates RCHO, which is also regenerated through the recycling of RCOOH by LuxC and LuxE. In *V. fischeri*, LuxG re-reduces FMN, although some bioluminescent bacteria lack *luxG* and other routes of recycling FMN back to the $FMNH_2$ substrate are available. The *lux* genes encoding these proteins are clustered and often in the order *luxCDABEG*.

Scientists have long puzzled over the costs of generating bioluminescence[7]. For example, LuxAB can comprise 5% of the protein in bright cells, and ATP is hydrolysed to regenerate RCHO. Moreover, the consumption of oxygen and reducing equivalents might compete with energy recovery from aerobic respiration. Before the genetics and biochemistry of bacterial bioluminescence were well understood, scientists noticed that undefined dim or dark mutants frequently arose during prolonged culture, and they speculated that lacking some natural selection to maintain luminescence, cells discarded it as energetically too expensive. With the advantage of modern genetics, we found that a *ΔluxCDABEG* mutant, which is completely dark, does indeed outcompete its wild-type isogenic parent[8].

**Figure 1.** Bioluminescent bacteria on Petri plates. First grade students at Barrow Elementary School, Athens GA, inoculated a *Photobacterium* strain on to Petri plates, generating these glowing works of art.

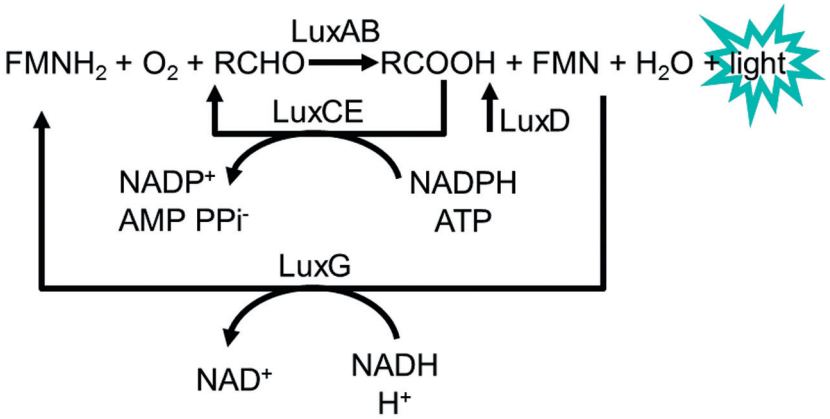



**Figure 2.** The Lux proteins that generate bacterial bioluminescence. See main text for details.

## The light switch

Given the costs of bioluminescence, bacteria carefully regulate when they induce *lux* gene expression, and the way they do so radically changed how we view the social lives of bacteria. In 1970, scientists in the Hastings laboratory published their seminal observations that

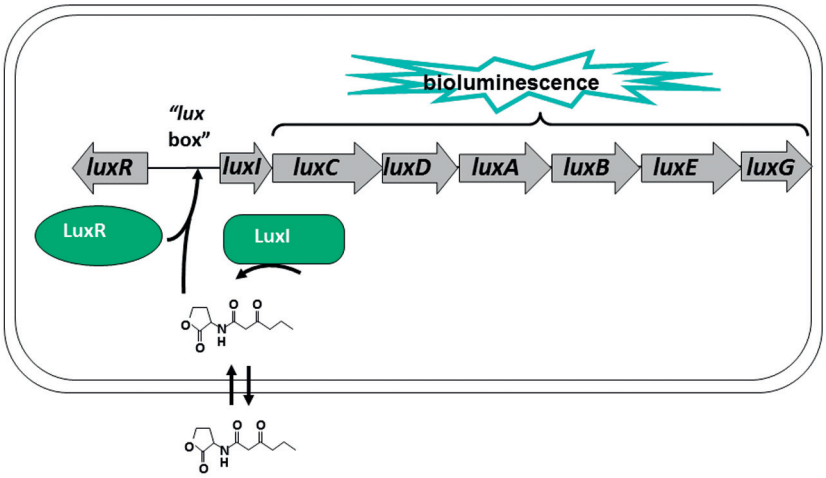



**Figure 3.** The *lux* genes and their pheromone-mediated regulation. The *luxICDABEG* operon includes genes required for luminescence as well as *luxI*, which encodes a pheromone synthase. When the pheromone produced by LuxI, 3OC6-HSL, accumulates to a sufficient concentration, it combines with LuxR, binds a '*lux* box' sequence, and stimulates *lux* operon expression.

dilute cells are dark, but they induce luminescence upon achieving higher population densities[9,10]. Thus the behaviour of individual cells was different if they were alone or in a crowd, and their mob mentality was to produce bioluminescence. Such behaviour made intuitive sense, because the light generated by a single bacterial cell, small as they are, could not be detected by any known biological system. Only in a group could their bioluminescence be visible.

Over the next 15 years, the mechanism of this cell-density-dependent regulation was elucidated (Figure 3). In *V. fischeri*, the *luxCDABEG* genes are expressed in an operon with *luxI*, which encodes a pheromone synthase. This operon is divergently transcribed from *luxR*, which encodes the cognate pheromone-dependent transcriptional activator[11]. LuxI synthesizes *N*-3-oxo-hexanoyl homoserine lactone (3OC6-HSL), which can diffuse between cells. Once 3OC6-HSL reaches a certain threshold concentration, at high cell density, it combines with LuxR and together they bind at a '*lux* box' sequence and activate transcription of *luxICDABEG*. Add 3OC6-HSL to dilute cells, and they induce luminescence.

3OC6-HSL was called 'autoinducer', and the entire regulatory scheme was cleverly dubbed 'quorum sensing' to convey that the behaviour was only undertaken when a sufficient number of individuals were present to do business. Although the precise mechanisms vary between bioluminescent bacteria, pheromone-mediated control of the *lux* genes was a consistent theme. The fact that there were distinct ways of accomplishing a similar end, an indicator of convergent evolution, added to the intrigue, and a field gelled around the term 'quorum sensing'.

## Shedding light on bacterial sex and violence

The history of bacterial pheromone signalling is an excellent example of basic research having unanticipated reach. For a time, the field of microbiology considered the phenomenon captivating and worthy of study, but perhaps also a bit esoteric – an odd quirk of glowing marine bacteria. Peptide pheromones in Gram-positive bacteria were elucidated at about the same time, and other reports of similar signalling trickled out, but the real scope of bacterial pheromone signalling did not unfold until the early to mid-1990s. Once researchers began looking in earnest, they found bacterial pheromones widespread, often underpinned by LuxI and LuxR homologues at least in the proteobacteria. Both plant and animal pathogens co-ordinate attacks on hosts in essentially the same way *V. fischeri* controls bioluminescence. Conjugative transfer of plasmid DNA between cells, a mechanism for spreading antibiotic resistance, is often pheromone controlled as well. Strategies have emerged for combating bacteria by thwarting their signalling. For example, transgenic plants that destroy bacterial pheromones are resistant to rot-causing pathogens[12]. The esoteric quirk of bioluminescent bacteria is now in textbooks and forms the basis for biotech start-ups.

## The plot thickens

By the time bacterial pheromone signalling became a staple of microbiology textbooks it was already clear that the story being told in those books is simplistic. It is true that pheromone accumulation as cell density increases is a hallmark of these systems, but the concept portrayed of pheromones as census-taking molecules glosses over regulatory complexities. Arguably, the term 'quorum sensing', which helped to popularize the field and to explain it at the same time, has unfortunately also reinforced this simplistic view. As it turns out, pheromone concentration does not follow a simple correlation with cell density and is instead highly context dependent for multiple reasons. For example, *V. fischeri* isolated from the Hawaiian bobtail squid are a thousand times brighter in the host than in culture, even at equivalent cell density.

Feedback loops are one reason that context, and not just cell density, matters. Alert readers may have noted that the 3OC6-HSL product of LuxI stimulates more LuxI production, and this positive feedback loop can lead to hysteresis, such that pheromone concentration is partly a function of whether the system has recently been stimulated[13]. Positive feedback is a remarkably common feature among bacterial pheromone systems, and it is not clear why this should be so if the purpose is to sense population density. Some have argued that it might reduce cell–cell variability or 'noise' during a population-wide response, and this may be true, but

recent technologies allowing detection of gene expression, and even bioluminescence, from single cells has shown considerable heterogeneity in responses. Positive feedback may have another role.

## Decoding bacterial languages

Context is also important in bacterial pheromone signalling because the expression of pheromone synthases and receptors is typically, if not always, regulated. The effects are more evident or dramatic in some systems than others, but responses to environmental cues control pheromone signalling. Consequently, the pheromones are potentially transmitting information about the environment. Moreover, positive feedback can amplify regulatory inputs into the system, so that information could be transmitted about environment at a distance, allowing a group response to a condition experienced by a subpopulation[14] (Figure 4). Thus, although pheromone signalling may allow bacteria to ask 'how many of us are here', it could also allow bacteria to ask 'what's it like over there' with communication only possible if there are enough bacteria to pass the signal along.

The idea that pheromone signalling is density-dependent yet communicating other information might explain the common observation of bacteria using multiple pheromones. *V. fischeri* produces three known pheromones: 3OC6-HSL , *N*-octananoyl homoserine lactone and 'AI-2', which is presumably a furanosyl borate diester (as it is in *V. harveyi*)[15]. Through interconnected circuitry, these pheromones all ultimately regulate bioluminescence[16]. Researchers have posed the question, why have multiple pheromone systems if their purpose is to monitor cell density? One suggestion is that multiple systems could allow more robust, homogenous and fine-tuned responses to cell density; however, that begs the question of why regulate these different systems in response to different environmental conditions? An alternative possibility is that bacteria are saying different things to each other with distinct pheromones.

To understand whether bacteria are communicating across populations about microenvironments, we must figure out the conditions that modulate pheromone signalling and observe the bacteria 'talking' in environments where these conditions vary. Much of the groundwork has already been done. Several recurrent themes have emerged across different systems. Carbon source, iron availability, redox conditions, among others often control pheromone systems. More research along these lines is warranted, but perhaps a greater challenge is to listen in on what bacteria are saying in ecologically relevant and potentially heterogeneous environments. We can't know if bacteria are asking 'what's it like over there' by growing them in shake flasks. Using fluorescent reporters, we found that *lux* transcription in *V. fischeri* varied over across microenvironments of the squid light organ[17], and similar *in vivo* approaches promise to tell us about gene expression in more natural contexts for the bacteria.

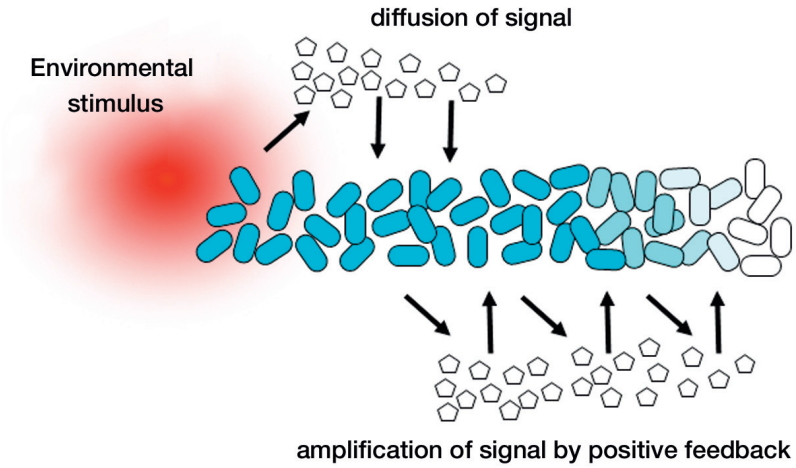
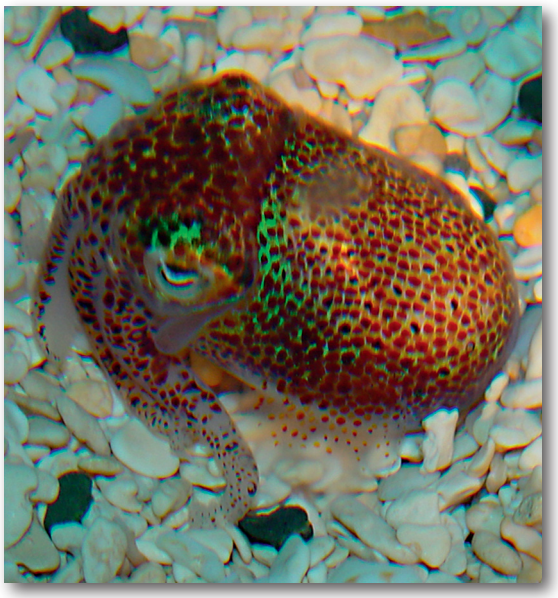



**Figure 4.** Regulation of pheromone signalling could co-ordinate group activities in a heterogeneous environment. An environmental cue stimulates bioluminescence and pheromone production (pentagons) in a group of cells, leading to diffusion of pheromone away from the stimulatory environment, inducing bioluminescence in other cells. Positive feedback then amplifies the response to the initial cue. The result of controlling pheromone synthesis environmental regulation combined with positive feedback is that a group response to a stimulus can be co-ordinated, even including cells that are not themselves in a stimulatory environment.

## Can regulation tell us anything about why cells make light in the first place?

Studying the regulation of bioluminescence uncovered a world of bacterial communication, but major questions about bioluminescence still remain. Among these is, what purpose does bioluminescence serve for the bacteria? The answer may vary for different bioluminescent bacteria. For symbionts such as *V. fischeri*, one explanation is that by helping their hosts, and receiving nutrients in return, they derive benefit. The Hawaiian bobtail squid is thought to use bioluminescence as camouflage[18], and the squid also grow and excrete *V. fischeri*, apparently resulting in higher populations of the bacterium[19]. However, that observation can't explain the entire benefit of luminescence. In contrast with *lux* genes impairing growth in culture, bioluminescence helps *V. fischeri* colonize the squid. Dark mutants are driven out [8,20,21]. Although the squid can perceive light and potentially sanction a dark infection, the light from nearby wild-type strains cannot save the dark mutants. There must be some aspect of the light organ environment that makes light production advantageous.

Several theories have been proposed to explain how luminescence can be advantageous[7], and regulation of



Hawaiian bobtail squid *Euprymna scolopes*

the genes is informative in differentiating between them. Some have suggested that in bioluminescent bacteria DNA damage induces luminescence, so that light can stimulate photolyase-mediated DNA repair, but that model cannot explain the colonization advantage of bright *V. fischeri* cells[22].

Alternative models focus on the Lux system's consumption of oxygen and reducing equivalents. One school of thought posited that luminescence benefits cells by burning excess reducing power to support fermentative growth; however, the ArcA/ArcB regulatory system and others seem to regulate in the opposite manner, with luminescence repressed in response to more reducing conditions. Seemingly more consistent with such regulation, several researchers have proposed that luminescence might benefit cells by consuming oxygen and protecting against oxidative stress. For example, luciferase might drive down ambient or intracellular oxygen concentration, either attenuating a host oxidative burst or rendering cells more resistant to oxidative stress. Luciferase's high affinity for $O_2$ ($K_m$=~35 nM) seems consistent with such a function. Moreover, the requirement of a 'quorum' to induce luminescence might reflect that lone dilute cells have little hope of affecting [$O_2$], and that only a concerted effort could be of any use.

## Light organ bioluminescence as an anti-oxidant?

Multiple lines of evidence indicate that *V. fischeri* is exposed to oxidatively stressful conditions in the host light organ, although the importance of oxidative stress and reactive oxygen species (ROS) in the symbiosis and

**Table 1.** Elements of *V. fischeri* oxidative stress response. P, proteomic analysis of adult light organ exudate; S, symbiotic defect; T, transcriptomic analysis of adult light organ.

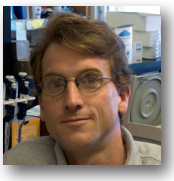
| Gene | Predicted function | Evidence of symbiotic role* |
|---|---|---|
| **Sensors of ROS** | | |
| VF_1974 | $H_2O_2$ inducible-transcriptional regulator | |
| *oxyR* | $H_2O_2$ inducible-transcriptional regulator | |
| *yjiE* | Hypochlorite-inducible transcriptional regulator | |
| **Scavengers of ROS** | | |
| *sodB* | Superoxide dismutase, Fe | P |
| *yfeX* | Iron-dependent peroxidase | |
| *bcp* | Thioredoxin-dependent peroxidase | P |
| *ahpC* | Alkyl hydroperoxide peroxidase | P |
| *yhjA* | Cytochrome *c* peroxidase | |
| *katA* | Catalase | PS |
| | Thioredoxin peroxidase | T |
| *tagD* | Lipid hydroperoxide peroxidase | |
| VF_A0890 | Thioredoxin peroxidase | P |

*Phenotypes based on references [23–25]

its relation to luminescence await further investigation. High levels of host-derived halide peroxidase have been detected in different symbiotic microenvironments, where it presumably functions to convert $H_2O_2$ and chloride ions into the potent oxidant HOCl. Similarly, nitric oxide has been detected in symbiotic tissues. Each of these ROS is toxic, and may keep symbiont populations in check.

*V. fischeri* appears well equipped to resist such stress (Table 1). The *V. fischeri* genome encodes components of putative $H_2O_2$, HOCl and superoxide stress responses. Moreover, the predicted $H_2O_2$ sensors and scavengers appear to be redundant, suggesting that this microbe is highly adapted to $H_2O_2$ stress. Mutational studies will be informative about the symbiotic importance of these systems. For example, a catalase mutant, which lacks the ability to detoxify $H_2O_2$, loses $H_2O_2$ resistance in culture and competitive fitness in the symbiosis, suggesting that *V. fischeri* is exposed to antimicrobial concentrations of at least $H_2O_2$ in the light organ[23]. Proteomic and transcriptional studies demonstrated that other bacterial oxidative stress-response genes, including some encoding predicted ROS scavengers and repair systems, are highly induced in the symbiosis[24,25]. Mutational study of these genes should likewise provide valuable information into their role and contribution in symbiosis and oxidative stress response.

If indeed bioluminescence acts to ameliorate oxidative stress, then the effects of losing other oxidative stress responses may be exacerbated by additional *lux* mutations. Such mutants should be tested both by challenge with antimicrobial ROS and in the light organ symbiosis. Although the importance of bioluminescence in symbiotic *V. fischeri* may not be representative of the role played by the *lux* genes in all bioluminescent bacteria, its symbiosis does afford a rare opportunity for laboratory-based experimentation on bacterial cells making light in an ecologically relevant context. ■



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## References


1. Harvey, E.N. (1952) Bioluminescence. Academic Press, New York
2. Harvey, E.N. (1957) A history of luminescence from the earliest times until 1900. American Philosophical Society, Philadelphia
3. Dunlap, P.V. and Kita-Tsukamoto, K. (2006) in The Prokaryotes (Dworkin, M., ed.), pp. 863–892 Springer, New York
4. Stabb, E.V. (2006) in The *Vibrio fischeri–Euprymna scolopes* light organ symbiosis (Thompson, F.L., Austin, B. and Swings, J., eds), pp. 204–218, ASM Press, Washington D.C.
5. Stabb, E.V. and Visick, K.L. (2013) in The Prokaryotes (Rosenberg, E., DeLong, E.F., Stackebrandt, E., Lory, S. and Thompson, F., eds) 497–532, Springer, New York
6. Hastings, J.W. and Nealson, K.H. (1977) Annu. Rev. Microbiol. **31**, 549–595
7. Stabb, E.V. (2005) ASM News **71**, 223–229
8. Bose, J.L., Rosenberg, C.S. and Stabb, E.V. (2008) Arch. Microbiol. **190**, 169–183
9. Hastings, J.W. and Greenberg, E.P. (1999) J. Bacteriol. **181**, 2667–2668
10. Nealson, K.H., Platt, T. and Hastings, J.W. (1970) J. Bacteriol. **104**, 313–322
11. Engebrecht, J., Nealson, K. and Silverman, M. (1983) Cell **32**, 773–781
12. Dong, Y.H., Wang, L.H., Xu, J.L., Zhang, H.B., Zhang, X.F. and Zhang, L.H. (2001) Nature **411**, 813–817
13. Williams, J.W., Cui, X., Levchenko, A. and Stevens, A.M. (2008) Mol. Syst. Biol. **4**, 234
14. Septer, A.N. and Stabb, E.V. (2012) PLoS ONE **7**, e49590
15. Chen, X., Schauder, S., Potier, N. et al. (2002) Nature **415**, 545–549
16. Stabb, E.V., Schaefer, A., Bose, J.L. and Ruby, E.G. (2008) in Chemical Communication Among Bacteria (Winans, S.C. and Bassler, B.L., eds), pp. 233–250, ASM Press, Washington D.C.
17. Dunn, A.K., Millikan, D.S., Adin, D.M., Bose, J.L. and Stabb, E.V. (2006) Appl. Environ. Microbiol. **72**, 802–810
18. Stabb, E.V. and Millikan, D.S. (2009) in Defensive Mutualism in Microbial Symbiosis (White, Jr, J.F. and Torres, M.S., eds), pp. 85–98, Taylor and Francis, Boca Raton
19. Lee, K-H, Ruby, E.G. (1994) Appl. Environ. Microbiol. **60**, 1565–1571
20. Koch, E.J., Miyashiro, T.I., McFall-Ngai, M.J. and Ruby, E.G. (2013) Mol. Ecol., doi:10.1111/mec.12474
21. Visick, K.L., Foster, J., Doino, J., McFall-Ngai, M. and Ruby, E.G. (2000) J. Bacteriol. **182**, 4578–4586
22. Walker, E.L., Bose, J.L. and Stabb, E.V. (2006). Appl. Environ. Microbiol. **72**, 6600–6606
23. Visick, K.L. and Ruby, E.G. (1998) J. Bacteriol. **180**, 2087–2092
24. Schleicher, T.R., Nyholm, S.V. (2011) PLoS ONE **6**, e25649
25. Wier, A.M., Nyholm, S.V., Mandel, M.J. et al. (2010) Proc. Natl. Acad. Sci. U.S.A. **107**, 2259–2264