

Shedding Light on the Bioluminescence “Paradox”

Although luminescence provides host squids with obvious advantages, how does it benefit light-producing bacteria?

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The fascinating biochemistry, genetics, and cell density-dependent regulation of bacterial bioluminescence provoke a challenging question. What good is it to bioluminescent bacteria?

There may be no single answer to this seemingly simple question. However, two recent advances shed new light on the problem. First, studies of the symbiosis between the bioluminescent bacterium *Vibrio fischeri* and the Hawaiian bobtail squid bring an ecologically relevant niche for a bioluminescent bacterium into focus under the discerning lens of controlled laboratory experimentation. Second, progress in understanding the genetics of *V. fischeri*, including genomic sequencing of a squid symbiont, is enabling researchers to analyze how luminescence integrates into the physiology of this bacterial species and to test specific hypotheses about what advantage light production confers on the bacteria.

Understanding how bioluminescence aids *V. fischeri* in a squid light organ will likely not be the final word on how bioluminescence benefits bacteria. Differences among bioluminescent bacteria intimate that this ancient system confers varied selective advantages. For example, “cryptically luminescent” *Vibrio* pathogens, such as *Vibrio salmonicida*, produce luciferase, the enzyme responsible for bioluminescence, but little or no aldehyde substrate, and may use luciferase to form “dark reaction” hydrogen peroxide as a host-damaging virulence factor. In contrast, some strains of *Vibrio logei* produce an accessory Y1 protein that shifts their emitted light to a yellow wavelength, which may

have special significance for this bacterium. Bioluminescence offers many such puzzles, and it is unlikely a single solution will solve them all.

Nonetheless, it is an exciting moment in bioluminescence research, with recent advances offering the promise of answering the longstanding question, “how can bioluminescence help bacteria?” Although researchers learned nearly a century ago that luminescence reduces oxygen and that symbiotic bacteria inhabit the light organs of squids, what was unimaginable until very recently is our ability to analyze the *V. fischeri* genome sequence and to combine this knowledge with the ability to genetically manipulate these bacteria and observe them under controlled laboratory conditions in the ecologically relevant environment of a natural squid host.

Biochemistry and Genetics of *V. fischeri* Bioluminescence

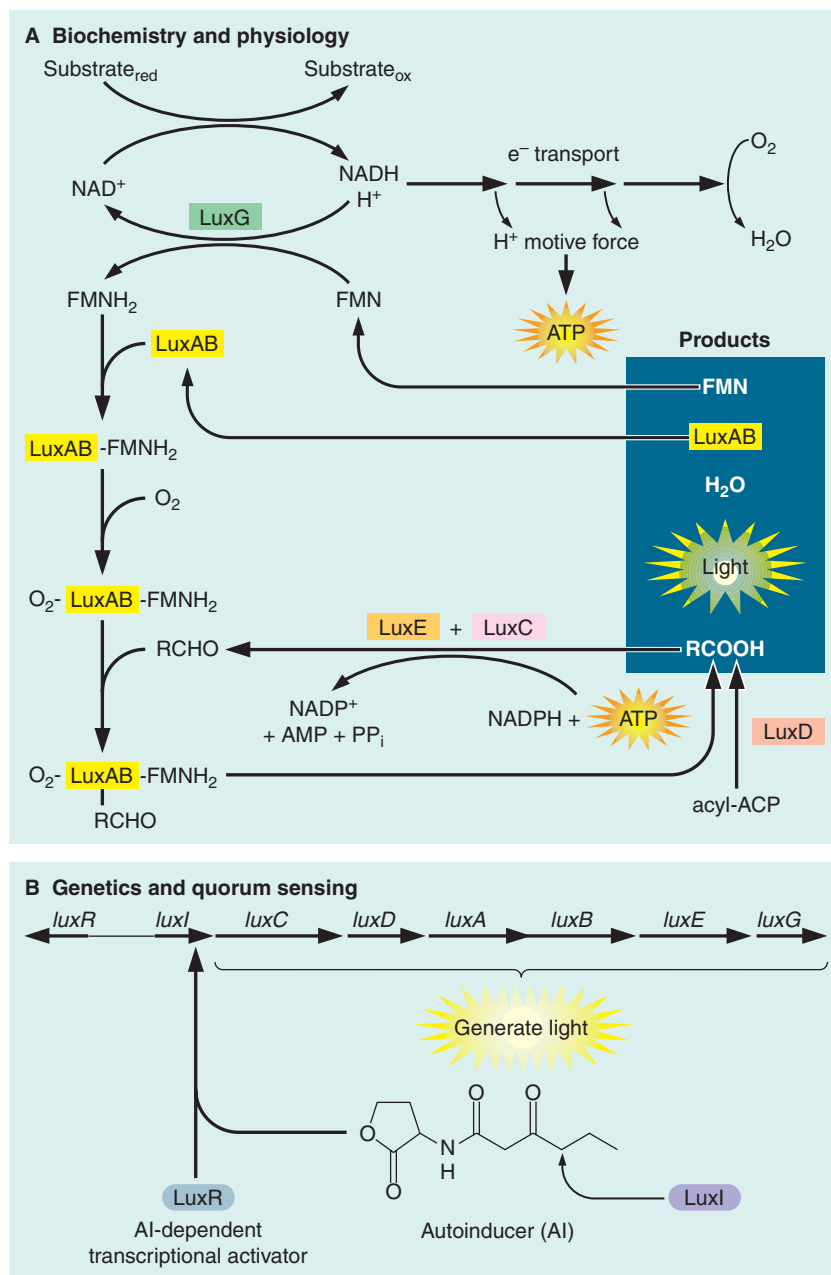
In bacteria such as *V. fischeri*, light is generated by an enzyme, luciferase, that contains two proteins, designated LuxA and LuxB (Fig. 1). LuxAB sequentially binds FMNH₂, O₂, and an aliphatic aldehyde (RCHO) that are converted to an aliphatic acid, FMN, and water. In turn, they are released from the enzyme with the concomitant production of light (Fig. 1A). Additional proteins, LuxC, LuxD, and LuxE, are responsible for (re)generating the aldehyde, while another protein, LuxG, shuttles reducing power from NAD(P)H to FMN to (re)generate FMNH₂.

In *V. fischeri*, the *luxC*, *luxD*, *luxE*, and *luxG* genes flank *luxA* and *luxB*. These genes are cotranscribed with *luxI*, while *luxR* is adjacent

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FIGURE 1



V. fischeri bioluminescence. (A) Biochemistry and physiology. (B) Genetics and quorum sensing.

to the other *lux* genes but not transcribed with them (Fig. 1B). Together LuxI and LuxR underlie a well-characterized quorum-sensing regulatory circuit, in which LuxI generates an autoinducer (AI) that interacts with LuxR to stimulate *lux* transcription. The expression of *lux* is stim-

ulated when intracellular AI concentration exceeds a threshold. Thus, light is produced at high cell densities, such as during growth in a squid light organ, but not when planktonic cells are growing at low densities.

Bioluminescence at First Analysis Appears To Be a Drag on Cell Energy

Despite having a good understanding of the biochemical and genetic mechanics of bioluminescence, researchers remain uncertain over what its selective advantage is to bacteria. In particular, the apparent costs in energy to cells from bioluminescence make its existence appear paradoxical.

In addition to the sizable biosynthetic cost in producing the Lux proteins, generating light consumes both biochemical reducing power and oxygen, seemingly competing for substrates with aerobic respiration, which recovers energy through electron transport (Fig. 1A). Furthermore, energy stored as ATP is consumed in regenerating the aldehyde substrate (Fig. 1A).

Indications that bioluminescence hinders cultured cells date at least as far back as E. Newton Harvey's work at Princeton University in the early 1900s. Those findings were extended by J. Woodland (Woody) Hasting at Harvard University and his collaborators, who showed that some relatively darker mutants outgrew their brighter parents. Similarly, Paul Dunlap, now at the University of Michigan, characterized bright mutants of *V. fischeri* that grew more slowly than do their relatively dim parent.

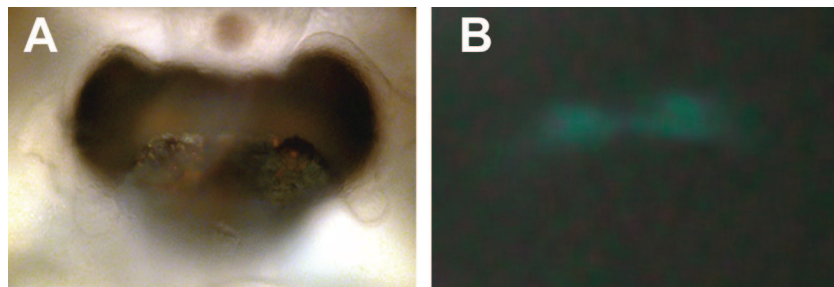
In 1980, Ken Nealson and David Karl, currently at the University of Southern California and the University of Hawaii, respectively, also found that cells

expend appreciable energy for luminescence. However, these two researchers did not find that bioluminescence slows growth rates.

These apparently inconsistent findings are difficult to interpret. Part of the explanation for the inconsistencies is that the best technology



FIGURE 2



Symbiotic bioluminescence. Light organs of *E. scolopes* juveniles under white light (left panel) or lit from within by bioluminescent *V. fischeri* symbionts (right panel). It is thought that adult *E. scolopes* use this ventrally directed luminescence to obscure their silhouette. Solid bars are $\sim 100 \mu\text{m}$.

of the day required comparisons of nonisogenic strains, analyses of undefined pleiotropic mutants, or induction of luminescence by AI, which also regulates non-*lux* genes. Recently, however, Grzegorz Wegrzyn's group at the University of Gdansk in Poland used defined strains to show that luminescence indeed slows the growth of bacteria in cultures. Specifically, he finds that a *luxA* mutant of *V. harveyi* outcompetes its isogenic parent. Similarly, we find that a *luxCDABEG* deletion mutant of *V. fischeri* outcompetes its isogenic wild-type parent in mixed culture when AI is present. Thus, at least under some conditions, light production slows bacterial growth.

Presumably, however, luminescence confers an advantage to bacteria in some settings. For instance, bioluminescent bacteria naturally inhabit and illuminate the light-emitting organs of animals such as the Hawaiian bobtailed squid, *Euprymna scolopes* (Fig. 2). Some biologists argue that, in such associations, "what's good for the host is good for the symbiont." In this specific case, *E. scolopes* apparently uses *V. fischeri*'s luminescence to elude its predators, suggesting that increased host fitness offsets the cost of bioluminescence to the bacteria, because the host "pays" the bacteria back with nutrients.

Even so, without other constraints, dark mutants theoretically still should outcompete their bright compatriots within a light organ, if forming only a minority population. However, available evidence indicates that such dark "cheaters" are not found in symbiont populations, suggesting luminescence yields additional advantages.

Squids Provide a Means for Scrutinizing Bioluminescence under Controlled Conditions

A major advance for bioluminescence research came when Margaret McFall-Ngai and Edward Ruby, who now are at the University of Wisconsin, Madison, brought the *V. fischeri*-*E. scolopes* symbiosis into the laboratory. Although several other hosts of bioluminescent bacteria cannot be bred in captivity, *E. scolopes* is an exception. In a series of ecological studies, Ruby and his collaborators found that *V. fischeri* in Hawaii are specifically adapted to *E. scolopes*, and they are more abundant in waters inhabited by the host.

Later, Karen Visick at Loyola University in Chicago, Ill., provided evidence that bacteria derive an advantage from bioluminescence in this symbiosis. Specifically, although a *luxA* mutant colonizes *E. scolopes*, this mutant (unlike its parental strain) does not persist well. One possibility is that this mutant is attenuated because of detrimental effects from expressing the full set of LuxCDBEG proteins in the absence of a functional LuxA. However, we tested an in-frame *luxCDABEG* deletion mutant and came up with a result equivalent to Visick's, namely poor persistence of this mutant in the squid. Thus, although bioluminescence slows *V. fischeri* growth in culture, it enhances the bacterium's colonization of the squid light organ. How?

How Bacteria May Benefit from Being Luminescent

Researchers have proposed several hypotheses to explain how bioluminescence could confer advantages to light-producing bacteria (see table). Several of these ideas downgrade the importance of luminescence itself, describing it as little more than an eye-catching distraction. Rather, some researchers argue that the important event occurs when luciferase consumes oxygen or biochemical reducing power within the bacterial cell. It may seem wasteful and thus counterintuitive to burn reducing power and oxygen without maximizing proton motive force and ATP generation; however, this is not unprecedented. Respiratory chains that are rel-

actively uncoupled from proton pumping help maintain redox balance, and in instances of “respiratory protection” they consume oxygen rapidly enough to protect oxygen-sensitive cytoplasmic proteins even when bacteria are in an aerobic environment.

This still leaves open the question of which is the more important reactant to “burn,” oxygen or biochemical reducing power? Many researchers believe the relevant reactant is oxygen, although for different reasons. For example, animal-associated bioluminescent bacteria may consume oxygen to keep it away from the host. Depriving the nearby host epithelium of oxygen could attenuate the animal’s ability to produce antimicrobial oxygen radicals, or it could generate a low-oxygen environment that facultative bacterial cells are better suited to cope with than are obligately aerobic host cells. On the other hand, bioluminescence may depress intracellular oxygen concentrations in the bacteria, either to increase resistance to oxidative stress in general or to protect specific oxygen-sensitive enzymes. Proponents of these explanations point to the relatively high affinity of luciferase for oxygen as evidence for bioluminescence driving down oxygen levels.

Other scientists believe that the relevant reactant is reducing power. The reducing power for bioluminescence comes indirectly from the NADH pool, and luciferase could therefore serve to recycle NAD⁺ cofactor. Jean-Jacques Bourgois and his collaborators at the Université Catholique de Louvain in Belgium recently bolstered this argument with evidence that reductant flows through luciferase only when respiration is saturated. They speculate that luminescence becomes important for symbiotic bacteria when the other primary electron sink, biomass production, becomes limited by spatial constraints—for example, in the confines of a light organ.

Despite such plausible benefits from burning oxygen or reducing power, the importance of producing light itself cannot be dismissed. Cheryl Whistler at the University of New Hampshire and Margaret McFall-Ngai propose that cryptochrome photoreceptors allow squid to detect bioluminescent symbionts and impose sanctions on dark bacteria. This model has evolutionary appeal in that it explains how the host

Table 1. How bioluminescence may benefit bacteria directly

Possible benefit	Relevant process	Mode of action
Suffocate the host	O ₂ consumption	Luminescence deprives nearby host epithelium of O ₂ , attenuating reactive oxygen species production
Holding their breath	O ₂ consumption	Luminescence lowers bacterial intracellular O ₂ , protecting O ₂ -sensitive enzymes and increasing resistance to oxidative stress
Redox sink	NAD ⁺ regeneration	Growth conditions lead to buildup of NADH, and luminescence scavenges O ₂ to burn the excess reductant
DNA repair	Light production	Luminescence stimulates light-dependent photolyase-mediated DNA repair in an otherwise dark environment
Avoid sanctions	Light production	Host distinguishes light-producing cells from dark ones and punishes the latter

ensures that it receives light from the symbiosis. How animals could distinguish and selectively sanction dark bacterial cells that are mixed with bright ones is not known, but finding cryptochromes in the light organ merits further investigation.

Yet another proposal is that bacterial bioluminescence stimulates DNA repair mediated by photolyase, an enzyme that uses visible-light energy to fix pyrimidine dimers. According to Wegrzyn and his colleagues, when *V. harveyi* is mutated by UV irradiation and then placed in the dark, the survival of *lux* mutants is attenuated. Whether this treatment reflects an ecologically relevant condition is unresolved, but DNA repair by luciferase and photolyase could be important even if something other than UV light damages the cellular DNA.

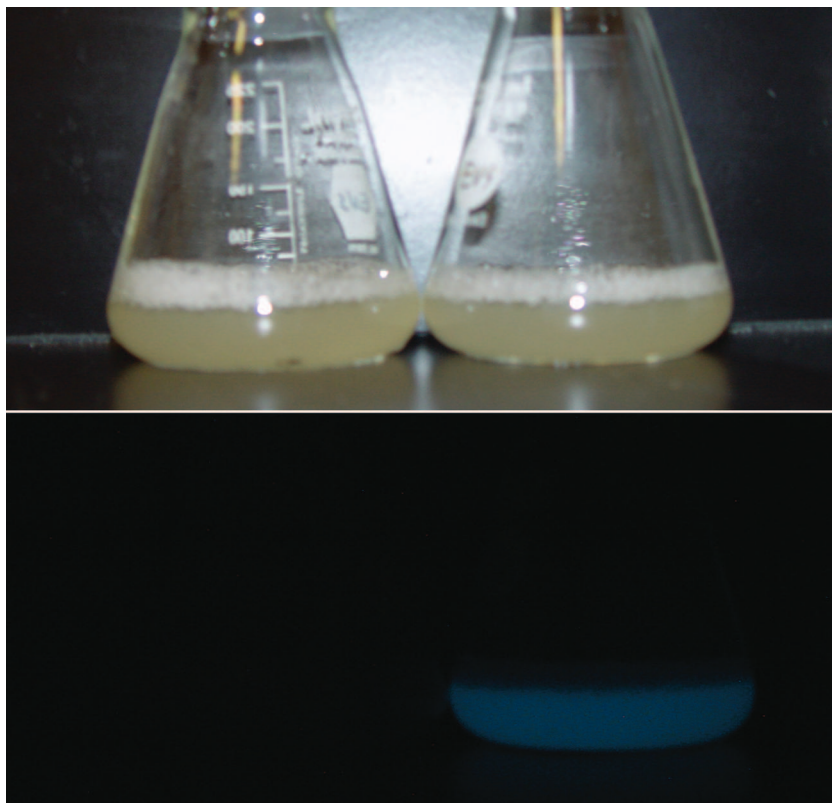
Wegrzyn and his collaborators also report a connection between the *lux* system and resistance to oxidative stress, although an aldehyde-deficient dark mutant is also resistant. Luciferase without aldehyde catalyzes a “dark reaction,” partially reducing oxygen to hydrogen peroxide, which might prime oxidative stress responses in this mutant.

Genetics and Genomics Shedding Light on Role of Bioluminescence in Bacteria

Genetic approaches should help us to evaluate which among the proposed beneficial roles of bioluminescence are valid. Bioluminescence helps *V. fischeri* to colonize *E. scolopes*, and this symbiosis can be manipulated by establishing it



FIGURE 3



Enhanced bioluminescence in an *arcA* mutant of *V. fischeri*. Culture flasks of wild type strain ES114 and its $\Delta arcA$ mutant illuminated (top panel) or in the dark (bottom panel).

with genetically altered bacteria under controlled conditions. Such genetic manipulations have become fairly standard, and include directed mutations, expression studies using reporters such as *lacZ* or green fluorescent protein, and complementation analyses using a native *V. fischeri* plasmid. The above hypotheses for the importance of luminescence provide numerous predictions that can be tested using such genetically engineered strains.

Meanwhile, knowledge of the *V. fischeri* genome sequence, which contains 4.3 million base pairs, is providing new ways to unravel the importance of bioluminescence in this bacterium. First, it provides a ready means for identifying and mutating specific genes to begin testing current hypotheses about the metabolic effects of luminescence. For example, if luminescence stimulates photolyase-mediated DNA repair, and this reflects the symbiotic benefit of luminescence, then *V. fischeri* mutants lacking this gene should

be attenuated in their ability to colonize *E. scolopes*. Genome analyses reveal one photolyase homologue, and we are now generating a mutant to test its symbiotic phenotype. Although such questions could be posed before genomic sequence information was available, genomics streamlines the process immensely.

Analysis of the *V. fischeri* genome also allows us to broadly assess metabolic pathways and to determine how bioluminescence fits into an integrated physiological matrix. In particular, we can predict which pathways might complement luminescence under specific scenarios. For example, suppose that symbiotic *V. fischeri* are growing exclusively on peptides and building up excess NADH under low-oxygen conditions, depending on luminescence to scavenge remaining oxygen and regenerate NAD⁺. Under these conditions, how would *V. fischeri* generate energy? What other pathways would be regenerating NAD⁺? If this “electron sink” model is correct, such pathways should be expressed during colonization of the host, and a *lux* mutation would make the cells more reliant on other NAD⁺ recycling pathways.

Genomic analyses suggest that *V. fischeri* would use certain amino acids to generate ATP and acetate, and that cells could recover NAD⁺ by producing lactate or succinate. This corresponds well with a 1942 report by Michael Doudoroff that 95% of the products of anaerobic peptone catabolism by *V. fischeri* were acetate, lactate, and succinate. If NADH buildup were problematic during growth on peptides, serine catabolism might be especially useful because serine deaminase does not reduce NAD⁺ in generating pyruvate. Genomic analysis suggests that the serine deaminase gene in *V. fischeri* is coexpressed with a functional homologue of LuxG. The genome also reveals pathways of anaerobic respiration that would be important if appropriate terminal electron acceptors were present. All these genes and their regulatory sequences are available for mutational, reporter gene, or microarray analyses.

Genome analysis provides other useful and

unexpected information as well. For example, it is often argued that luciferase has a higher affinity for oxygen than respiration does, and therefore might be useful in decreasing ambient O₂. However this is based on assumptions that *V. fischeri* possesses a low-O₂-affinity CyoABCD-type terminal oxidase and on measurements of respiration in cells grown at relatively high O₂, where a high-affinity respiratory system might not be induced. Genomic analyses reveal that *V. fischeri* lacks CyoABCD but possesses a homolog of the high-O₂-affinity CydAB-type terminal oxidase and a terminal oxidase similar to the very-high-O₂-affinity FixN of *Rhizobium loti*, which has greater affinity for oxygen than does luciferase. Such sequence similarities cannot tell us whether luminescence or respiration has higher O₂ affinity, but they do suggest that it would be worthwhile to reexamine whether luminescence or respiration is more important for setting ambient O₂.

The genome sequence is also helpful in determining how the *lux* genes are regulated, which will likely provide clues regarding the function-

al significance of luminescence. For instance, *V. fischeri* contains conserved regulatory pathways that may modulate *lux* expression in response to substrate availability. Among these, the ArcAB two-component regulatory cascade is especially intriguing.

In *Escherichia coli*, ArcB senses redox state and, under reduced conditions, phosphorylates ArcA, which acts as a DNA-binding transcriptional regulator. In *V. fischeri* ArcA and ArcB may act as a regulatory switch if luminescence is expressed to counteract oxidative stress or excess reductants. Because ArcA is activated by phosphorylation under reduced conditions, ArcA-P may stimulate *lux* transcription if luciferase functions as an electron sink, or to repress *lux* if luciferase counteracts oxidative stress. Our preliminary data indicate that ArcA-P represses *lux* in culture (Fig. 3) but that *lux* control by the ArcAB system is derepressed in the host. If correct, this would suggest that luciferase does not benefit *V. fischeri* in the host squid by burning excess reductant, but may instead be expressed to counteract oxidative stress.

ACKNOWLEDGMENTS

I thank Jeffrey Bose for helping generate the figures, and Edward Ruby for many useful discussions. I was supported in this work by a CAREER award from the National Science Foundation (MCB-0347317), and by a Junior Faculty Research Grant from the University of Georgia Research Foundation. Genome information was provided by the *Vibrio fischeri* Genome Project, at <http://ergo.integratedgenomics.com/Genomes/VFI>, supported by the W. M. Keck Foundation. I wish to express my admiration and appreciation of the many scientists who have contributed to the field of bioluminescence, especially those who are not credited in this article or included in the Suggested Reading section below. E. Newton Harvey introduced the bibliography of his amazingly thorough book *Living Light*, published in 1940, by apologizing for its incompleteness; "The literature on luminescence is so vast that it is impossible to include (all) the important. . . papers." The task has not gotten any easier in the intervening 65 years!

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