13 Luminous Bacteria

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Abstract
Luminous bacteria are those bacteria that carry the *lux* genes, genes that code for proteins involved in light production. Many luminous bacteria emit light at high, easily visible levels in laboratory culture and in nature, and the phenomenon of light emission has generated interest in these bacteria for over 125 years. Luminous bacteria are especially common in ocean environments where they colonize a variety of habitats, but some species are found in brackish, freshwater, and terrestrial environments. This chapter, which begins with an historical perspective, summarizes current understanding of the biochemistry and genetics of bacterial light emission, the taxonomy and phylogenetics of light-emitting bacteria, the evolutionary origins and hypothesized physiological and ecological functions of bacterial luminescence, the distributions and activities of these bacteria in nature, their symbiotic interactions with animals and especially with marine fishes, and the quorum sensing regulatory circuitry controlling light production at the operon level. This chapter concludes with information on the isolation, cultivation, storage, and identification of luminous bacteria.

Introduction and Historical Perspective

Luminous bacteria are those bacteria that carry *lux* genes, at a minimum *luxA* and *luxB*, the genes coding for bacterial luciferase, either as vertically inherited genes or genes naturally acquired by horizontal transfer. Most of the currently known luminous bacteria express the *lux* genes and produce light at high, readily visible levels in laboratory culture (*Fig. 13.1*) or in nature. Not all *lux* gene-carrying bacteria, however, produce levels of light visible to the human eye. To date, luminous bacteria have been found in only three closely related *Gammaproteobacteria* families, *Vibrionaceae*, *Enterobacteriaceae*, and *Shewanellaceae*, and most species are members of *Vibrionaceae*. Most luminous bacteria are facultatively aerobic, but two, *Shewanella hanedai* (Jensen et al. 1980) and *Shewanella woodyi* (Makemson et al. 1997), are respiratory. Additional and detailed information on the metabolism, physiology, morphology, and ecology of these bacterial groups and individual species can be found in Baumann and Baumann (1981), Baumann et al. (1984), Farmer and Hickman–Brenner (1992), Boemare et al. (1993), Forst et al. (1997), and Urbanczyk et al. (2007). Bacterial luminescence is one of several evolutionarily distinct forms of bioluminescence, an attribute of a wide diversity of eukaryotic organisms (Hastings 1995; Widder 2010).

The ability of certain bacteria to produce light has been known since 1875, when Pflüger (1875) related the luminescence coming from the slime of fish to bacteria present in the slime (Harvey 1957; Robertson et al. 2011). Many earlier observations suggest the presence of luminous bacteria and knowledge of their existence. During the 1700s and 1800s, various animal products (such as meats, fish, and eggs), the decaying bodies of marine and terrestrial animals, and even human wounds and corpses, were reported to emit light (Harvey 1940, 1952). Many

E. Rosenberg et al. (eds.), *The Prokaryotes – Prokaryotic Physiology and Biochemistry*, DOI 10.1007/978-3-642-30141-4_75,

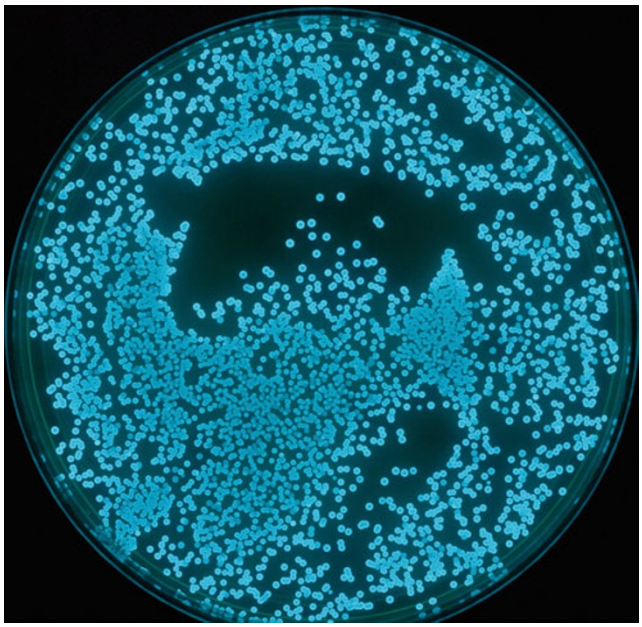

Fig.13.1
***Bacterial bioluminescence*. Colonies of *P. mandapamensis* from the light organ of the cardinalfish *Siphamia versicolor* (Perciformes: Apogonidae) are shown growing on LSW-70 agar plates. The plate was photographed in the dark by the light produced by the bacteria**

years before those observations and long before bacteria and the oxygen dependence of bacterial luminescence were known, Boyle (1668) demonstrated that the "uncertain shining of fish," the light coming from decaying fish, required air. Indeed, encounters with luminous objects and substances extend back to the beginnings of recorded history in Greece and China (Harvey 1957), and they continue in modern times to be causes of concern and wonder. Many of these encounters can be attributed to the saprophytic or pathogenic growth of luminous bacteria on or in marine and terrestrial animals.

According to Harvey (1940), J. F. Heller in 1854 was the first to give a name, *Sarcina noctiluca*, to an organism suspected to be responsible for luminescence. Following Pflüger's work in 1875, other scientists working in the late 1800s and early 1900s isolated and named luminous bacteria, including "*Bacterium lucens*," "*Micrococcus phosphorescens*," "*Micrococcus pflügeri*," "*Bacillus phosphorescens*," and "*Bacterium phosphoreum*" (Neush 1879; Ludwig 1884; Fischer 1887; Molisch 1912; Dahlgren 1915; Zobell 1946; Harvey 1952, 1957; Robertson et al. 2011). Particularly notable among early researchers of bacterial luminescence was Martinus W. Beijerinck, a founder of general microbiology, who carried out research on the physiology of light-emitting bacteria and who coined the name *Photobacterium*, a genus within which he grouped all luminous bacteria (Beijerinck 1889a, b, 1891, 1916; van Iterson et al. 1940; Robertson 2003; Robertson et al. 2011). The recent revival and phylogenetic characterization of strains isolated by Beijerinck and stored in the 1920s (Figge et al. 2011) provide a direct link to the origins of general microbiology and the first studies of luminous bacteria.

Following these early studies at the end of the nineteenth and the beginning of the twentieth century, luminous bacteria were isolated from various habitats, the chemistry of bacterial light production and the culture requirements for growth and luminescence of the bacteria were characterized, and they were placed taxonomically as microbial systematics developed (e.g., Zobell and Upham 1944; Farghaly 1950; Johnson 1951). In the latter half of the twentieth century and continuing to date, taxonomic efforts have paralleled the growth of microbiology, incorporating the tools and knowledge developing from advances in biochemistry, physiology, and genetics (Baumann and Baumann 1977, 1981; Farmer and Hickman–Brenner 1992; Hastings and Nealson 1977, 1981; Hendrie et al. 1970; Nealson and Hastings 1992; Singleton and Skerman 1973). Presently, over 25 species of luminous bacteria are validly described (*Table 13.1*). Marine luminous species are found in *Aliivibrio*, *Photobacterium*, *Vibrio* (*Vibrionaceae*), and *Shewanella* (*Shewanellaceae*), and terrestrial light-producing species are members of *Photorhabdus* (*Enterobacteriaceae*) (Dunlap and Kita–Tuskamoto 2006; Urbanczyk et al. 2007, 2008; Ast et al. 2009; Dunlap 2009; Yoshizawa et al. 2009a, b; 2010a, b). Current understanding of the systematic relationships of luminous bacteria, as well as recent descriptions of new species, has utilized phylogenetic analysis of multiple, functionally independent housekeeping genes, including the 16S rRNA gene, *gyrB*, *pyrH*, and *recA*, among others (e.g., Ast and Dunlap 2005; Thompson et al. 2005; Ast et al. 2007b, 2009; Urbanczyk et al. 2007). Particularly useful for resolving the separate species status of closely related luminous bacteria is sequence analysis of the *lux* genes, *luxCDABE*, found to date in all luminous bacteria, due to their relatively rapid sequence divergence compared to most housekeeping genes (Ast and Dunlap 2004, 2005; Dunlap et al. 2004; Ast et al. 2007a; Urbanczyk et al. 2007). The description of several new species in the past few years (*Table 13.1*) suggests that many more species of luminous bacteria remain to be discovered. The advents of whole genome sequencing, metagenomics, and single-cell genomics and their application to luminous bacteria will undoubtedly provide additional insight into the systematics of luminous bacteria, the evolution of the bacterial luminescence system, and many other aspects of the biology of these bacteria.

Biochemistry of Bacterial Luminescence

Light emission in bacteria is catalyzed by luciferase, a heterodimeric protein of approximately 80 kD, composed of α (40 kDa) and β (37 kDa) subunits. Bacterial luciferase mediates the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic (fatty) aldehyde (RCHO) by O_2 to produce blue-green light according to the following reaction:

$$\mathbf{FMNH_2 + O_2 + RCHO \xrightarrow{\textit{luciferase}} FMN + H_2O + RCOOH + h\nu\ (490\ nm)}$$

In the luminescence reaction, binding of $FMNH_2$ by the enzyme is followed by interaction with O_2 to form a flavin-4a-hydroperoxide. Association of this complex with aldehyde forms

Table 13.1
Species and habitats of luminous bacteria

Species	Habitats[a]	Selected references
Marine		
Aliivibrio fischeri	Coastal seawater,	Boettcher and Ruby (1990), Fitzgerald (1977), Lee and Ruby (1992)
	Light organs of squid and fish	Reichelt and Baumman (1973), Ruby and Nealson (1976)
		Ruby and Nealson (1978), Ruby et al. (2005), Urbanczyk et al. (2007)
logei	Coastal seawater, sediment	Ast et al. (2009), Bang et al. (1978), Urbanczyk et al. (2007)
salmonicida	Tissue lesions of Atlantic salmon	Hjerde et al. (2008), Nelson et al. (2007), Urbanczyk et al. (2007)
sifiae	Coastal seawater	Ast et al. (2009), Yoshizawa et al. (2010a)
"thorii"	Light organs of squid	Ast et al. (2009), Fidopiastis et al. (1998)
wodanis	Coastal seawater, diseased farmed salmon, light organs of squid	Ast et al. (2009), Lunder et al. (2000), Urbanczyk et al. (2007)
Photobacterium aquimaris	Coastal seawater	Yoshizawa et al. (2009b)
damselae	Coastal seawater	Smith et al. (1991), Urbanczyk et al. (2008)
kishitanii	Light organs and skin of fish	Ast and Dunlap (2005), Ast et al. (2007)
leiognathi	Coastal seawater, light organs of fish	Boisvert et al. (1967), Dunlap et al. (2007), Dunlap et al. (2008), Fukasawa and Dunlap (1986), Fukasawa et al. (1988), Reichelt et al. (1977)
mandapamensis	Coastal seawater, light organs of fish	Hendrie et al. (1970), Kaeding et al. (2007), Reichelt and Baumman (1973), Urbanczyk et al. (2011b), Wada et al. (2006)
phosphoreum	Coastal and pelagic seawater	Ast and Dunlap (2005), Ast et al. (2007a), Baumann et al. (1980), Budsberg et al. (2003), Ruby and Morin (1978), Wimpee et al. (1991)
Candidatus		
Photodesmus katoptron[b]	Light organs of anomalopid fish	Haygood (1990), Hendry and Dunlap (2011), Wolfe and Haygood (1991)
Shewanella hanedai	Seawater and sediment	Jensen et al. (1980)
woodyi	Seawater and squid ink	Makemson et al. (1997)
Vibrio azureus	Coastal seawater	Yoshizawa et al. (2009a)
"beijerinckii"	Coastal seawater	Figge et al. (2011)
campbellii	Coastal seawater	Lin et al. (2010)
chagasii	Coastal seawater, surfaces and intestines of marine animals	Thompson et al. (2003), Urbanczyk et al. (2008)
harveyi	Coastal seawater, sediment	Gomez–Gil et al. (2004), O'Brien and Sizemore (1979), Reichelt and Baumman (1973), Ruby and Nealson (1978), Yetinson and Shilo (1979)
mediterranea[c]	Coastal seawater	Pujalte and Garay (1986), Ortiz Conde et al. (1989)
orientalis	Seawater, surface of shrimp	Yang et al. (1983)
sagamiensis	Coastal seawater	Yoshizawa et al. (2010b)
splendidus	Coastal seawater	Baumann et al. (1980), Nealson et al. (1993)
vulnificus	Coastal seawater, oysters	Oliver et al. (1986), Urbanczyk et al. (2008)
Brackish/Estuarine		
Vibrio cholerae	Estuaries, bays coastal seawater	Kaeding et al. 2007; Palmer and Colwell (1991), Ramaiah et al., (2000), Zo et al. (2009)
Terrestrial		
Photorhabdus asymbiotica	Human skin lesions	Farmer et al. (1989); Fisher–Le Saux et al. (1999), Peel et al. (1999)

Table 13.1 (continued)

Species	Habitats[a]	Selected references
luminescens	Insect larvae infected with heterorhabditid nematodes	Boemare et al. (1993), Fisher–Le Saux et al. (1999)
temperata	Insect larvae infected with heterorhabditid nematodes	Fisher–Le Saux et al. 1999

[a]Representative habitats of luminous strains are listed.
[b]Candidatus name, not cultured (Hendry and Dunlap 2011).
[c]Ability of this species to luminescence is not well established; the single strain reported as luminous (Pujalte and Garay 1986) may not be available.

a highly stable intermediate, the slow decay of which results in oxidation of the $FMNH_2$ and aldehyde substrates and the emission of light. Quantum yield for the reaction has been estimated at 0.1–1.0 photons. The reaction is highly specific for $FMNH_2$, and the aldehyde substrate in vivo is likely to be tetradecanal. $FMNH_2$ is provided by the activity of an NAD(P)H-flavin oxidoreductase (flavin reductase). Synthesis of the long-chain aldehyde is catalyzed by a fatty acid reductase complex composed of three polypeptides, an NADPH-dependent acyl protein reductase (r, 54 kDa), an acyl transferase (t, 33 kDa), and an ATP-dependent synthetase (s, 42 kDa). The complex has a stoichiometry of $r_4s_4t_{2–4}$, and its activity is essential for the production of light in the absence of exogenously added aldehyde. Luciferases from different species of luminous bacteria exhibit substantial amino acid residue and nucleotide sequence identity (Meighen and Dunlap 1993; Dunlap et al. 2007), consistent with a common evolutionary origin of luminescence in bacteria. For references and detailed information on the biochemistry of bacterial light production, the reader is directed to reviews by Hastings (1995), Lee et al. (1990), Hastings et al. (1985), Meighen (1988; 1991), Meighen and Dunlap (1993), and Wilson and Hastings (1998).

Species and Phylogeny of Luminous Bacteria

Over 25 species of luminous bacteria are validly described at this time (◉ *Table 13.1*). Taxonomically, luminous bacteria are members of six of genera in three *Gammaproteobacteria* families: *Vibrionaceae*, *Enterobacteriaceae*, and *Shewanellaceae* (◉ *Fig. 13.2*) To date, no luminous strains belonging to other families have been reported. Most luminous species are members of *Aliivibrio*, *Vibrio*, and *Photobacterium* in *Vibrionaceae*. Detailed phylogenetic analysis has shown that most extant luminous members of *Vibrionaceae* acquired their *luxCDABE* genes vertically, with only a few cases of acquisition by intraspecies horizontal transfer from members of *Vibrionaceae*, whereas luminous members of *Enterobacteriaceae* and *Shewanellaceae* apparently acquired their *lux* genes by horizontal transfer from members of *Vibrionaceae* (Urbanczyk et al. 2008). These considerations, and others described below, suggest that the *luxCDABE*-based luminescence system of bacteria arose just once evolutionarily, apparently in an ancestor of *Vibrionaceae* (Urbanczyk et al. 2008).

Several new species of luminous bacteria have been described in the past few years (◉ *Table 13.1*, ◉ *Fig. 13.2*). These include *Aliivibrio sifiae* (Ast et al. 2009; Yoshizawa et al. 2010a), *Photobacterium kishitanii* (Ast et al. 2007a), *Photobacterium aquimaris* (Yoshizawa et al. 2009b), *Candidatus* Photodesmus katoptron (Hendry and Dunlap 2011), *Vibrio azureus* (Yoshizawa et al. 2009a), and *Vibrio sagamiensis* (Yoshizawa et al. 2010b). Recent studies have also revealed the presence of luminous strains of species not previously reported as luminous, that is, *Vibrio campbellii* (Lin et al. 2010), *Vibrio vulnificus* (Urbanczyk et al. 2008), and *Vibrio damsela* (Urbanczyk et al. 2008). With respect to *V. campbellii*, a recent genomic analysis has revealed strain ATCC BAA-1116 (aka BB120), previously classified as *Vibrio harveyi* and studied intensively for quorum sensing control of luminescence and other cellular functions in this species (e.g., Bassler et al. 1993; Waters and Bassler 2005; Long et al. 2009), is actually a member of *V. campbellii* (Lin et al. 2010). Furthermore, newly recognized clades, for example, *Aliivibrio* "*thorii*" and *Vibrio* "*beijerinckii*" (Ast et al. 2009; Figge et al. 2011), have been identified, and formal description of these and other new species is under way. In most cases, taxonomic identification has followed cultivation-based detection of light emission; most of the bacteria listed in ◉ *Table 13.1* grow and emit light in laboratory media. However, several luminous bacteria, bioluminescent symbionts of anomalopids (flashlight fish) and ceratioids (deep-sea anglerfish), are known that have not been cultured; these bacteria are members of *Vibrionaceae* but are divergent from known species of luminous bacteria (Haygood 1990, 1993; Haygood and Distel 1993). Very recently, luminous bacteria symbiotic with the anomalopid fish *Anomalops katoptron* were characterized phylogenetically and assigned *Candidatus* status as a new *Vibrionaceae* genus and species, Photodesmus katoptron (Hendry and Dunlap 2011).

Most luminous strains isolated from natural habitats group taxonomically as members of well-recognized species that typically are considered to be luminous (◉ *Table 13.1*). However, luminescence often is not a uniformly consistent phenotype of even these luminous species (e.g., Wollenberg et al. 2011) or their genera. Nonluminous species of *Vibrio* are well known and are more common than luminous strains (e.g., Baumann and Baumann 1981), and several nonluminous species of *Photobacterium* and *Aliivibrio* have been found, some of which apparently lack *lux* genes (Dunlap and Ast 2005; Urbanczyk et al. 2011a). Furthermore, strains of *Photorhabdus luminescens* symbiotic with entomopathogenic nematodes have been found that do not produce light and lack genes necessary for light

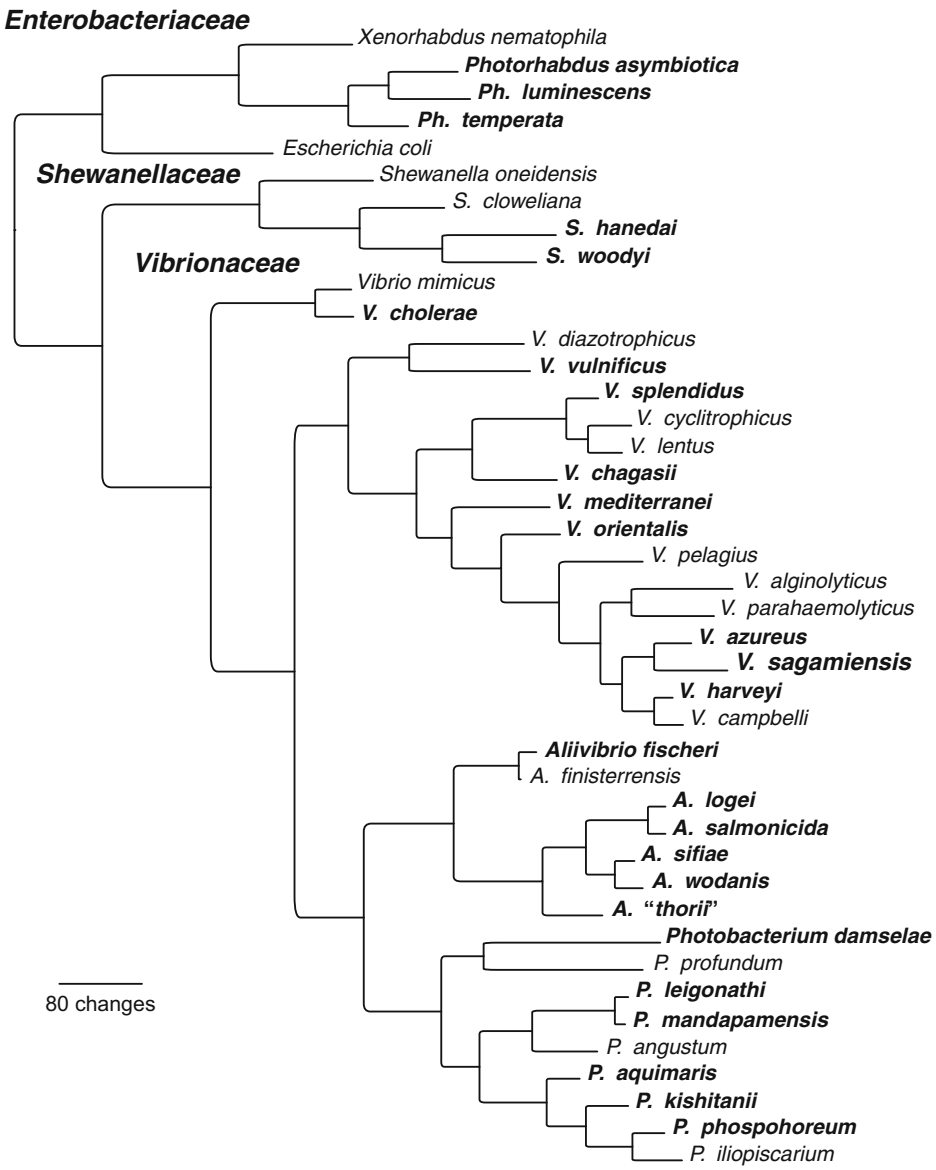

Fig. 13.2

***Phylogeny of luminous bacteria*. The analysis, parsimony implemented in PAUP*, is based on sequences of the 16S rRNA and *gyrB* genes. Luminous species (in boldface) are found in three families, *Vibrionaceae*, *Shewanellaceae*, and *Enterobacteriaceae*. These families contain many more nonluminous species than shown here. Also, recently identified luminous bacteria, for example, *Vibrio "beijerinckii"* (proposed name) (Figge et al. 2011) and *Candidatus* Photodesmus katoptron (Hendry and Dunlap 2011), and additional species whose descriptions are underway, are not shown**

production (Akhurst and Boemare 1986; Forst and Nealson 1996). In addition, strains luminous on primary isolation often become dim or dark in laboratory culture (Nealson and Hastings 1979, 1992; Akhurst 1980; Silverman et al. 1989), and some species that grow well in laboratory culture at room temperature, that is, *A. logei* and *S. hanedai*, typically produce readily visible light only when grown at cooler temperatures. In some cases, that is, luminous bacteria infecting crustaceans (Giard and Billet 1889b) and strains of *A. fischeri* symbiotic with the Hawaiian sepiolid squid, *Euprymna scolopes* (Boettcher and Ruby 1990), the bacteria produce a high level of light in their natural habitat but produce little or no light when grown in laboratory culture.

Adding to this complexity, *V. cholerae*, generally considered to be a nonluminous species, has many luminous strains (e.g., Kaeding et al. 2007; Zo et al. 2009), and many of the nonluminous strains of this species carry *lux* genes that apparently are not expressed in laboratory culture (Palmer and Colwell 1991; Ramaiah et al. 2000). In addition, bacteria identified as related to *V. harveyi* and *V. cincinnatiensis* carry the *lux* genes but have been found to have *lux* gene mutations that result in a dark phenotype (O'Grady and Wimpee 2008). Furthermore,

luminous strains of three other species generally known as nonluminous, *Vibrio vulnificus*, *Vibrio chagasii*, and *Photobacterium damselae*, recently were identified (Urbanczyk et al. 2008). This substantial variation in the incidence of luminous strains within a species has implications for understanding the evolutionary origins of bacterial luminescence and its patterns of inheritance, as described in sections that follow. It should be noted that the presence of luminescence strains has likely been overlooked in many species. Routine use of cooler temperatures (10–20 °C) for growth and examination, and utilization of conditioned media, inducers, and luciferase substrates (Fidopiastis et al. 1999), along with the application of probes for *luxA* and other *lux* genes (Wimpee et al. 1991), and full sequence-based characterization of the *lux* operons of new luminous bacteria will undoubtedly lead to a more complete understanding of the species diversity of bacteria able to produce light. A further complication in gaining a more comprehensive understanding of the diversity of luminous bacteria is that descriptions of new species of luminous bacteria often lack detailed information on the *lux* genes, relationships to type strains, or detailed phylogenetic analysis (e.g., Yoshizawa et al. 2009b, 2010b). In this regard, characterization of multiple newly isolated strains, the use of multiple independent loci, and the use of type strains and other key strains are imperatives in new species descriptions for revealing the otherwise hidden species diversity of luminous bacteria (e.g., Ast et al. 2009).

The Bacterial Luminescence Operon

The genes coding for the α- and β-subunits of bacterial luciferase, *luxA* and *luxB*, respectively, are part of the *lux* operon, *luxCDABE*, which is present in the genomes of all luminous bacteria examined to date as a conserved, contiguous, and coordinately expressed set of genes (Fig. 13.3). The *luxC*, *luxD*, and *luxE* genes, respectively, code for the r, s, and t polypeptides of the fatty acid reductase complex that synthesizes and recycles aldehyde substrate for luciferase. The *lux* operons of most bacteria also contain *luxG*, which codes for a flavin reductase (Lin et al. 1998; Meighen and Dunlap 1993; Nijvipakul et al. 2008; Swartzman et al. 1990a). The absence of *luxG* from the *lux* operon of *Ph. luminescens* apparently is compensated for by the activity of a flavin reductase activity coded for by an *Escherichia coli fre*-like gene, homologs of which are found in various luminous bacteria (Zenno et al. 1992, 1994; Zenno and Saigo 1994). Several species of *Photobacterium* bear an additional *lux* operon gene, *luxF*, between *luxB* and *luxE*. The *luxF* gene, coding for a nonfluorescent flavoprotein, is apparently specific to *Photobacterium*, as it is not present in the *lux* operons of *Aliivibrio*, *Photorhabdus*, *Shewanella*, or *Vibrio* species (Fig. 13.3), but it has been secondarily lost in *Photobacterium leiognathi* (Ast and Dunlap 2004). The LuxF protein might function in the luminescence system by scavenging an inhibitory side product of the luciferase reaction (Moore and James 1995), but it is not necessary for light production even in those *Photobacterium* species that normally carry this gene (Kaeding et al. 2007). In the examined species of luminous *Photobacterium*, the *lux* operon genes are followed, without a transcriptional stop or other regulatory sites, by genes involved in the synthesis of riboflavin, *ribEBHA*, the products of which presumably function in generating $FMNH_2$, a substrate of luciferase (Lee and Meighen 1992; Lee et al. 1994; Lin et al. 2001; Sung and Lee 2004; Ast et al. 2007b). We refer to this gene arrangement as the *Photobacterium lux-rib* operon (Fig. 13.3). Strains of *P. phosphoreum* lack one of the *rib* genes, *ribE*; the gene presumably was lost in the divergence from an ancestral *Photobacterium* that gave rise to this species. The presence of genes for synthesis of riboflavin as part of the *lux* operon might facilitate light production by ensuring coordinate synthesis of luciferase and substrates for the enzyme. In this regard, the *lux* operon of *V. campbellii* (previously classified as *V. harveyi*; Lin et al. 2010) contains *ribB*, coding for 3,4-dihydroxy-2-butanone 4-phosphate synthase, a key enzyme in riboflavin synthesis (referred to originally as *luxH*; Swartzman et al. 1990b) as the final gene, as does the *lux* operon of *Candidatus* Photodesmus katoptron (Hendry and Dunlap 2011). Furthermore, although *ribB* is not part of the *lux* operon of *A. fischeri*, its expression nonetheless is under the same quorum sensing control as the *lux* genes (Callahan and Dunlap 2000).

In addition to presence of *ribEBHA* genes in *Photobacterium* as part of the *lux-rib* operon, genes upstream of the *lux* operon contribute to luminescence and also show genus and species differences. In *Photobacterium mandapamensis*, for example, the *lux-rib* operon is preceded by *lumQ* and *lumP*, which form the lumazine operon. The function of *lumQ* is not yet known, although it might code for a DNA binding protein (Lin et al. 1995). LumP, a 21 kDa fluorescent accessory protein referred to as lumazine protein, functions to shift the emission wavelength of luciferase from blue-green (495 nm) to blue (475–486 nm) and enhance the intensity of light emission (Lee 1993; O'Kane et al. 1985,1991; Petushkov et al. 1996). LumP, which has been isolated from *P. phosphoreum* and strains called *P. leiognathi* (which actually are *P. mandapamensis*, see below) and also purified from *P. kishitanii*, contains a noncovalently bound fluorophore, 6,7-dimethyl-8-ribityllumazine, the immediate biosynthetic precursor of riboflavin (O'Kane et al. 1985; Sato et al. 2010; Small et al. 1980). In *P. leiognathi lumP* is not found, although approximately 200 nucleotides of the *P. leiognathi luxC–lumQ* intergenic region can be aligned to the *P. mandapamensis lumP* gene (Fig. 13.4; Ast et al. 2007b). The activity of the LumP protein apparently accounts for the blue-shifted luminescence of *P. mandapamensis* compared to *P. leiognathi*, one of the diagnostic traits distinguishing these two species (O'Kane et al. 1985, 1991; Lee 1993; Petushkov et al. 1996; Ast and Dunlap 2004; Kaeding et al. 2007). The genes flanking the *P. leiognathi* and *P. mandapamensis lux-rib* operons are homologous to a single contiguous region in nonluminous *P. angustum* (Lin et al. 1993, 1995, 1996a, b, 2001; Ast et al. 2007b).

In the examined *Aliivibrio* species, regulatory genes, *luxI* and *luxR*, which control transcription of the *lux* operon, precede or flank the *luxCDABEG* genes (Fig. 13.3). The *luxI* gene codes for an acyl-homoserine lactone (acyl-HSL) synthase

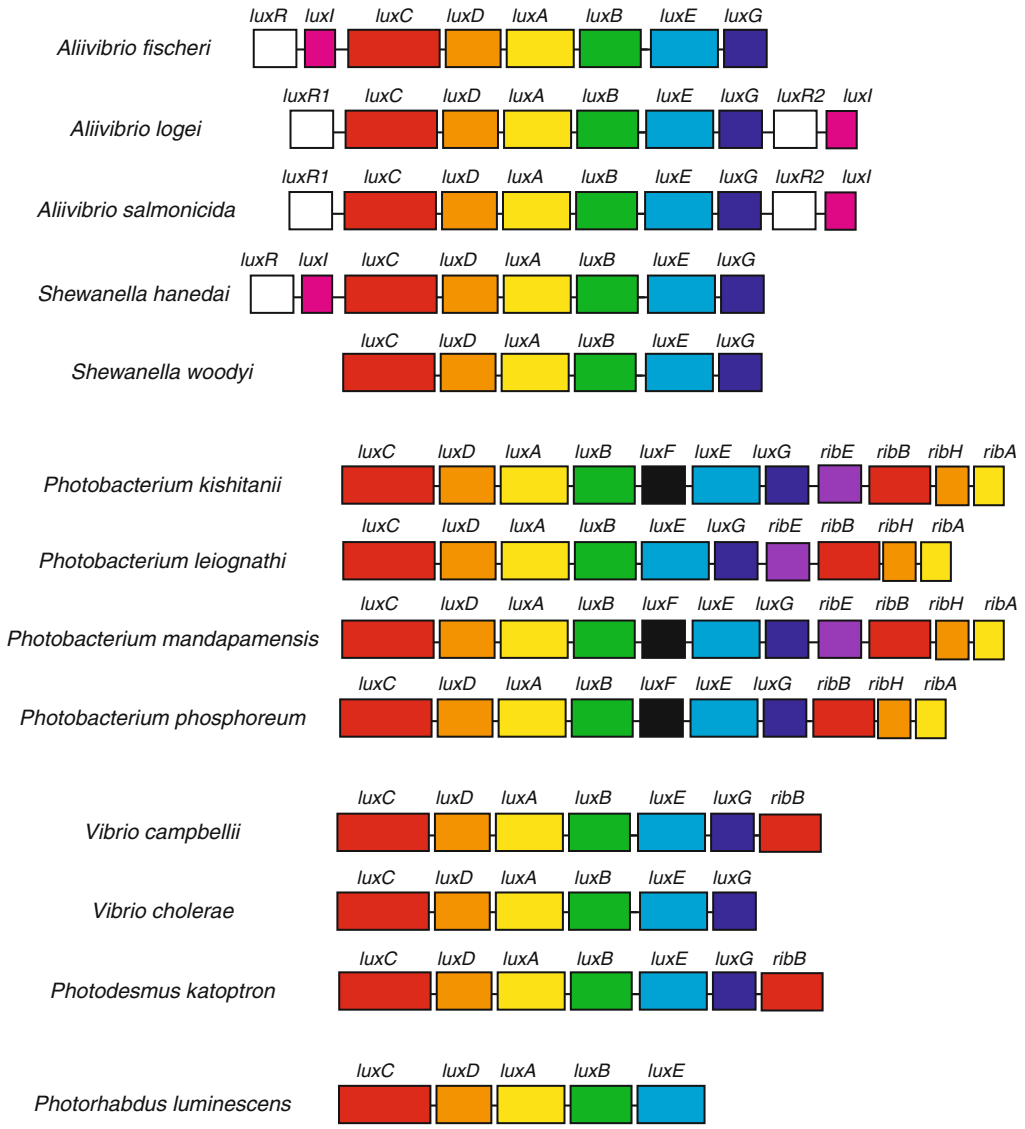

Fig. 13.3

***Genes of the lux operons of luminous bacteria*. Shown are the lux genes and the organization of lux operons for those bacteria for which complete lux operon sequence data are available. Contiguous genes of the luminescence operons of luminous bacteria are aligned to highlight commonalities and differences. Four distinct types of lux operons are evident based on commonalities of gene content, organization, and sequence similarity, (1) the *Aliivibrio/Shewanella* type, with *luxI/luxR* regulatory genes; (2) *Photobacterium* type, with *ribEBHA* genes; (3) the *Vibrio/Candidatus* Photodesmus type, with neither regulatory nor additional linked genes; and (4) the *Photorhabdus* type, composed of just the core *luxCDABE* genes**

(Schaefer et al. 1996), and *luxR* codes for a receptor protein that interacts with acyl-HSL to activate transcription of the *lux* operon (Engebrecht et al. 1983), as described in more detail below. In *Aliivibrio fischeri*, *luxI* is the first gene of the *lux* operon, and *luxR*, upstream of *luxI*, is divergently transcribed (◗ *Fig. 13.3*). The same gene arrangement is present in *Shewanella hanedai*, and this identity together with the high degree of *lux* gene sequence similarity in *S. hanedai* and *A. fischeri* has led to the suggestion that *S. hanedai* acquired its *lux* operon by horizontal transfer from *A. fischeri* or the ancestor of *A. fischeri* (Urbanczyk et al. 2008), as described in more detail below. In *Aliivibrio salmonicida*, a bacterium that requires exogenous addition of aldehyde to produce a high level of light (Fidopiastis et al. 1999), two *luxR* genes, homologous to *A. fischeri luxR*, flank the *lux* operon; a *luxI* gene also is present, divergently transcribed from the downstream *luxR* (◗ *Fig. 13.3*; Nelson et al. 2007; Hjerde et al. 2008). Very recently, the same arrangement of *luxI* and *luxR* genes as in *A. salmonicida* was identified in *Aliivibrio logei* (Manukhov et al. 2011). In contrast to *A. salmonicida*, however, *A. logei* does not require exogenous aldehyde to produce a high level of light (Manukhov et al. 2011); sequence comparison of the two operons identified mutations in

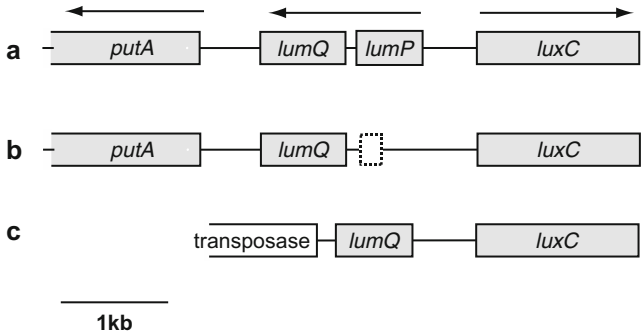

Fig. 13.4

***Region upstream of the lux operon in Photobacterium*. (a) The *lux-rib* operon is preceded in *P. mandapamensis* by *lumQ* and *lumP*. (b) For the *lux-rib*$_1$ operon of *P. leiognathi*, *lumQ* is present upstream and *lumP* is not found, although approximately 200 nucleotides of the *P. leiognathi luxC–lumQ* intergenic region can be aligned to the *P. mandapamensis lumP* gene sequence. (c) The region upstream of the *lux-rib*$_2$ operon of *P. leiognathi* contains *lumQ* and a transposase gene. For details, see the text and Ast et al. (2007b). The regions flanking the *lux-rib* operons of other *Photobacterium* species remain to be defined, but preliminary information for some species indicates differences from the arrangement shown here (Urbanczyk et al. unpublished data)**

luxD of *A. salmonicida* that presumably account for the exogenous aldehyde requirement of this species. Genes flanking the *lux* operons of other luminous *Aliivibrio* species (Table 13.1) apparently have not yet been identified.

The arrangement of genes flanking the *lux* operons of the examined *Vibrio* species differs substantially from that in *Photobacterium* and in *Aliivibrio* (Fig. 13.3). First, regulatory genes controlling transcription of the *lux* operon are not part of and are not adjacent to the *lux* operon in those *Vibrio* species examined; specifically, a *luxR* gene, which is not homologous to *A. fischeri luxR*, is not physically associated with the *lux* operon in *V. campbellii* (*V. harveyi*); the role of *luxR*$_{Vh}$ in the phosphorelay cascade controlling luminescence in this species is outlined below. Conservation of *luxCDABE* as a unit might reflect a need for close interaction of luciferase and fatty acid reductase proteins, based on coordinate regulation, to facilitate substrate generation necessary for efficient light production. However, it is not obvious what led to the genus-specific differences in the presence of genes flanking and contiguous with the *lux* operons of luminous members of *Aliivibrio*, *Photobacterium*, and *Vibrio*, three closely related genera of *Vibrionaceae*.

Genomes of Luminous Bacteria

The genomes of luminous *Vibrio* and *Photobacterium* species are similar in structure, overall size, and organization to other members of *Vibrionaceae*, with two chromosomes of unequal size and an overall size of approximately 4.5–5.4 Mb (Egan et al. 2005; Okada et al. 2005; Ruby et al. 2005; Vezzi et al. 2005; Reen et al. 2006; Lauro et al. 2009; Ast et al. 2007a; Urbanczyk et al. 2011a, b; Urbanczyk et al. unpublished data). The significance of this organization for members of *Vibrionaceae* is not yet known, but differences between the two replicons suggest that each chromosome carries out substantially different roles in the cell. More of the core (essential) genes are found on the large chromosome, whereas the small chromosome contains mostly lineage specific genes. Furthermore, gene content and position appear to be more highly conserved on the large chromosome than on the small chromosome (Reen et al. 2006). The small chromosome in members of *Vibrionaceae* nonetheless contains some essential genes (Reen et al. 2006), which might guarantee retention of the small chromosome during cell division (Egan et al. 2005). The origin of the small chromosome in *Vibrionaceae* remains unknown but has been hypothesized in *V. cholerae* to have originated from a plasmid that accumulated additional genes, including some genes transferred from the large chromosome (Egan et al. 2005). The apparent ubiquity of two chromosomes of unequal size in *Vibrionaceae* suggests that the small chromosome may have arisen in the ancestral lineage leading to *Vibrionaceae*.

In view of the different predicted roles for the large and small chromosomes in *Vibrionaceae*, it is significant that in luminous species for which complete sequences of both chromosomes are available, the *lux* operons are all located on the small chromosome. These species include *A. fischeri* (Ruby et al. 2005; Mandel et al. 2009), *A. salmonicida* (Hjerde et al. 2008), and *V. campbellii* (Lin et al. 2010). This pattern indicates that the *lux* genes have an accessory function, that is, they are not part of the core genome, a view that is consistent with the many nonluminous *Vibrionaceae* species and many nonluminous strains of luminous species, as noted above. Which chromosome, large or small, carries the *lux* genes is not yet known in *Photobacterium* species. A single chromosome is characteristic of *Enterobacteriaceae* and *Shewanellaceae*, and the *lux* genes in *Photorhabdus* and luminous *Shewanella* are chromosomal.

The genomes of luminous bacteria analyzed to date have been found to carry multiple rRNA operons. Specifically, the genome of *A. fischeri* carries 12 rRNA operons (Ruby et al. 2005), the *P. kishitanii* genome has eight or more (Ast et al. 2007a), the *P. mandapamensis* genome has six or more (Urbanczyk et al. 2011b), and the *Ph. luminescens* genome has seven (Duchaud et al. 2003; Wilkinson et al. 2009). A high copy number of rRNA operons may be an adaptation for a copiotrophic lifestyle and for rapid response to nutrient availability (Klappenback et al. 2000; Lauro et al. 2009). Taken together, the relatively large genome size and multiple rRNA operons of luminous bacteria and other members of *Vibrionaceae* may be adaptations for rapidly utilizing a wide range of different nutrients under feast-or-famine conditions.

Evolutionary Origin and Function of Bacterial Luminescence

The origin of bacterial luminescence has been of interest since the early days of microbiology. The natural presence of genes

necessary for producing light defines the luminous bacteria. The necessary genes, *luxA* and *luxB*, encoding the luciferase subunits, *luxC*, *luxD* and *luxE*, for the fatty acid reductase subunits, and *luxG*, encoding a flavin reductase, are consistently found together as a cotranscribed unit, *luxCDABEG*. The reason for this conservation of *lux* genes as a unit is not known, but it might relate to efficient light production; the contiguous presence of these genes as an operon might help promote the coordinated production of luciferase and substrates for luciferase, long-chain aldehyde and reduced flavin mononucleotide ($FMNH_2$). The conservation of these genes as a unit in nearly all luminous bacteria examined suggests that the *lux* operon arose just once in the distant past. Supporting this view, phylogenetic analysis demonstrates that the individual *lux* genes of different bacterial species are homologous, as was suggested by the high levels of amino acid sequence identities of the inferred Lux proteins. This homology implies that the bacterial *luxCDABEG* genes arose one time in the evolutionary past. The use by luciferase of oxygen as a substrate implies that this enzymatic activity originated after oxygenic photosynthesis by ancestors of modern-day cyanobacteria began to increase the level of O_2 on Earth, approximately 2.4 billion years ago, during the Great Oxidation Event. A marine origin for bacterial luminescence (Palmer and Colwell 1991; Dunlap 2009) seems likely because most species of luminous bacteria are marine (*Table 13.1*).

Seliger (1987) proposed that bacterial luminescence arose under ecological selection for light emission. A flavoprotein catalyzing fatty acid α-oxidation reactions with low chemiluminescent quantum yields is postulated to have mutated under hypoxic conditions to accept $FMNH_2$ as the flavin cofactor, generating a fortuitously high fluorescence yield, termed "protobioluminescence," via the 4a-hydroxy-FMNH product. This flavin-dependent, aldehyde-oxidizing protoluciferase produced sufficient light and with an appropriate emission spectrum, to be detected by phototactic organisms. Ingestion by visually cueing animals of particles colonized and made luminous by these early luminous bacteria presumably enhanced their reproduction by bringing them into the animal's nutrient-rich digestive system, ensuring the emitter's survival and thereby possibly leading to selection for more intense light output (Widder 2010). It is possible that early evolutionary steps leading to protoluciferase involved oxygen detoxification activity that permitted early anaerobic organisms to survive an increasingly aerobic environment (McElroy and Seliger 1962; Rees et al. 1998). An alternative hypothesis for the evolution of bacterial luciferase for DNA repair (Czyż et al. 2003) has been called into question (Walker et al. 2006).

A single gene was hypothesized to encode bacterial protoluciferase (O'Kane and Prasher 1992). Although a single-subunit protoluciferase, monomer or dimer, presumably would have differed somewhat from the modern-day luciferase α-subunit and therefore might have produced light, the inability of either of the extant α- or β-subunits alone to produce light in vitro or in vivo (Li et al. 1993) argues against the single-gene hypothesis. Alternatively, bacterial protoluminescence may have arisen following a gene duplication event that is postulated to have created *luxB* from *luxA* (Baldwin et al. 1979; O'Kane and Prasher 1992; Meighen and Dunlap 1993). Based on amino acid sequence identities, a tandem duplication of the ancestral *luxA* gene, followed by sequence divergence in the duplicated gene, is thought to have given rise to *luxB*, leading to the formation of the heterodimeric luciferase present in extant luminous bacteria. Similarly, a tandem duplication of *luxB* followed by loss of approximately 300 nucleotides coding for N-terminus amino acids is thought to have given rise to *luxF* in a luminescent ancestor of *Photobacterium*; this gene apparently was later secondarily lost in *P. leiognathi* (Baldwin et al. 1979; O'Kane and Prasher 1992; Meighen and Dunlap 1993; Ast and Dunlap 2004; Dunlap 2009).

Although the evolutionary origin of *luxA* and other bacterial luminescence genes remains obscure (Dunlap and Kita–Tuskamoto 2006), the conserved gene content and gene order of the *lux* operon in bacteria, *luxCDABEG*, and the high levels of *lux* gene and Lux protein amino acid sequence identities among luminous bacteria (e.g., Meighen and Dunlap 1993) leave little doubt of the homology of all presently known bacterial *lux* operons. Furthermore, the general congruence of phylogenies based on *lux* genes and other protein coding genes (and the 16S rRNA gene) (Urbanczyk et al. 2008) suggests that the *lux* operon is ancestral at least to *Aliivibrio*, *Photobacterium*, and *Vibrio*, and possibly to *Vibrionaceae*. The association of the fatty acid reductase genes, *luxCDE*, with *luxA* might have predated the *luxA* to *luxB* gene duplication event. Alternatively, the presence of ERIC sequences flanking *luxA* and *luxB* in *Ph. luminescens* (Meighen and Szittner 1992) might mark an insertion of the *luxAB* genes into the fatty aldehyde reductase operon during the evolution of the bacterial luminescence system. The origins and evolution of other luminescence genes are not well understood (O'Kane and Prasher 1992).

The evolution of bacterial luminescence system also involved recruitment of regulatory and other genes to the *lux* operon. The *lux* operons of certain *Aliivibrio* species contain two regulatory genes, *luxR* and *luxI* (*Fig. 13.3*), the protein products of which mediate a population density-responsive autoinduction, that is, quorum sensing. Recruitment of regulatory genes to the *lux* operon during evolution of *Aliivibrio* presumably enhanced quorum sensing control of luminescence (Dunlap 2009). Furthermore, as mentioned above, luminous *Photobacterium* strains carry genes involved in the synthesis of riboflavin, the *ribEBHA* genes, as part of the *lux-rib* operon (Lee et al. 1994; Ast et al. 2007b). Recruitment of the *rib* genes to the *lux* operon likely happened in an ancestor of *Photobacterium*, since other luminescent bacteria contain the *rib* genes elsewhere in the genome and not associated with the *lux* operon (e.g., Callahan and Dunlap 2000). An interesting exception to that general pattern is the presence of *ribB* initially named (*luxH*) as the last gene of the *lux* operon in *V. campbellii* (previously classified as *V. harveyi*; Lin et al. 2010).

The production of light consumes a substantial amount of energy, through the synthesis of Lux proteins and through their activity (Dunlap and Greenberg 1991). This energetic cost, which may explain the fact that luciferase synthesis is regulated in most luminous bacteria, suggests that activity of the

luminescence system plays an important role in the physiology and ecology of luminous bacteria. Most attention to what that role might be has focused on oxygen. One consideration is that, as noted above, the light-emitting reaction might have arisen evolutionarily as a detoxification mechanism, removing oxygen and thereby allowing an organism that is otherwise anaerobic to survive. Related to this possibility is that luciferase, as an oxidase, might function as a secondary respiratory chain that is active when oxygen or iron levels are too low for the cytoplasmic membrane-associated electron transport system to operate. This activity would allow cells expressing luciferase to reoxidize reduced coenzyme even when oxygen levels are low (Hastings and Nealson 1981; Hastings 1983; Nealson and Hastings 1992). Consistent with this view, growth of cytochrome-deficient luminous bacteria is dependent on induction of luciferase, limitation for iron stimulates light production, low oxygen levels promote the luminescence of some luminous bacteria, and luciferase synthesis can be induced under anaerobic conditions (Eberhard et al. 1979; Haygood and Nealson 1985; Makemson 1986; Makemson and Hastings 1982; Nealson and Hastings 1977, 1979). As an alternative to the electron transport system, the activity of luciferase in reoxidizing reduced coenzyme could permit cells of luminous bacteria in low oxygen habitats, such as in animal gut tracts, to continue to transport and metabolize growth substrates, thereby continuing to gain energy through substrate-level phosphorylation. Furthermore, light production presumably facilitates dissemination of luminous bacteria. The feeding of animals on luminous particles (decaying tissues, fecal pellets, and moribund animals infected by luminous bacteria), to which they are attracted, would bring the bacteria into the animal's nutrient-rich gut tract for additional rounds of reproduction followed by dispersal (Hastings and Nealson 1981; Nealson and Hastings 1992), and recent evidence supports this possibility (Zarubin et al. 2012). Alternatively, the function of the bacterial *lux* system might be to generate a halotolerant flavodoxin, with light emission an incidental consequence (Kasai 2006). Future studies may test and possibly provide additional support for these and other proposed functions for luminescence, such as a physiological role for luciferase activity in bioluminescent symbioses, but it is not yet clear what factors, physiological or ecological, actually select for the retention and expression of this energetically expensive enzyme system.

Horizontal Acquisition of the Bacterial *lux* Genes

Inheritance of the *lux* genes has been shown to be primarily vertical. However, some instances of acquisition by horizontal transfer have been identified (Ast et al. 2007; Urbanczyk et al. 2008). In the instances identified, horizontal acquisition of *lux* genes within *Vibrionaceae* has been found to be limited to species within the same genera, and no instance of the horizontally transferred genes replacing vertically inherited *lux* operons has been reported. In contrast to the proposal that horizontal gene transfer drives bacterial speciation (e.g., Gogarten et al. 2002; Ochman et al. 2000), horizontal acquisition of *lux* genes apparently has not led to phylogenetic divergence of the recipients (Urbanczyk et al. 2008). The predominant pattern of vertical inheritance of the *lux* genes, together with the fact that most species of luminous bacteria are members of *Vibrionaceae*, leads to the hypothesis that these genes arose in an ancestor of *Vibrionaceae*. The scattered incidence of luminous members in *Vibrionaceae*, with many nonluminous species and many species with nonluminous strains, indicates that the *lux* genes have been lost from many descendants of this putative ancestor (Urbanczyk et al. 2008; Dunlap 2009).

In *Photobacterium*, many strains of *P. leiognathi* carry two intact and apparently functional *lux-rib* operons in their genomes (Ast et al. 2007b). This situation represents an unusual case of natural merodiploidy in bacteria, the presence of two or more copies of the same gene or genes in the genome of a bacterium, because of the large number of genes involved and because the second operon did not arise by tandem duplication of the first. The two *lux-rib* operons are distinct in sequence and genomic location. One operon, *lux-rib*$_1$, is in the ancestral chromosomal location of the *lux-rib* operon in *P. leiognathi* and related bacteria. The other, *lux-rib*$_2$, is located elsewhere in the genome and is present in many but not all strains of *P. leiognathi*; it is flanked by genes coding for transposases, which suggests it can transfer between strains. Phylogenetic analysis indicates that the *lux-rib*$_1$ and *lux-rib*$_2$ operons are more closely related to each other than either is to the *lux* and *rib* genes of other bacterial species (Ast et al. 2007b). This finding rules out interspecies horizontal transfer as the origin of the *lux-rib*$_2$ operon in *P. leiognathi*; instead, *lux-rib*$_2$ apparently arose in the distant past within a lineage of *P. leiognathi* that either has not yet been sampled or has gone extinct.

Merodiploidy of the *lux-rib* operon in *P. leiognathi* also is the first instance of merodiploid strains of a bacterium having a nonrandom geographic distribution; strains bearing a single *lux-rib* operon are found over a wide geographic range, whereas *lux-rib* merodiploid strains have been found only in coastal waters of Honshu, Japan (Ast et al. 2007b; Urbanczyk et al. 2012b). The presence of multiple copies of each of the *lux* and *rib* genes might provide opportunities for sequence divergence and selection that could lead to the evolution of new gene functions in one or the other of the duplicate genes.

The *P. leiognathi lux-rib*$_2$ operon has also been found in two strains of *P. mandapamensis*, which also carry a normal *P. mandapamensis lux-rib* operon, and in a strain of *P. damselae*, a species not previously known to be luminous (Urbanczyk et al. 2008). Furthermore, evidence has been obtained indicating horizontal acquisition of the *lux* genes by a recently recognized species, *P. aquimaris* (Yoshizawa et al. 2009b; Urbanczyk et al. 2012a).

With respect to *S. hanedai* and *S. woodyi*, comparison of genes flanking the *lux* operons suggested that these species had acquired *lux* genes from a member of *Aliivibrio* (Kasai et al. 2007), a possibility confirmed through phylogenetic analysis (Urbanczyk et al. 2008). In *Photorhabdus* species as well, the

luxCDABE genes may have been acquired by horizontal gene transfer (Forst et al. 1997), possibly from an ancestor of *V. harveyi* (Meighen 1999). Differences between ecologically distinct strains of *Ph. luminescens* in the DNA flanking the *lux* operon (Meighen and Szittner 1992) raise the possibility that lateral transfer of the *lux* genes to this species occurred more than once (Forst et al. 1997). However, phylogenetic analysis of the *Photorhabdus lux* genes in the context of *Vibrionaceae* and *Shewanellaceae* sequences did not find support either for or against horizontal acquisition of the *lux* genes by *Ph. luminescens* (Urbanczyk et al. 2008). It is possible that substantial sequence divergence of the *lux* genes has occurred since their transfer to *Ph. luminescens*, thereby making problematic the identification of their source species.

Instances of the horizontal acquisition of *lux* genes have been identified also in *Vibrio* (Urbanczyk et al. 2008). The only known luminous strain of the human pathogen *V. vulnificus* (Oliver et al. 1986) apparently acquired its *lux* genes from *V. harveyi*, and in *V. chagasii*, a species not previously known to be luminous (Thompson et al. 2003), two luminous strains were identified and through phylogenetic analysis were shown to have acquired their *lux* genes apparently from *V. harveyi* and *V. splendidus*, respectively (Urbanczyk et al. 2008). A mechanism for these transfers, however, has not been proposed.

It should be noted that most species of *Vibrionaceae* lack the *lux* genes and therefore are nonluminous. Also, most strains of some luminous species, such as *V. cholerae*, are nonluminous. The low incidence of luminous species in the family suggests that the *lux* genes have been lost over evolutionary time from many of the lineages that have given rise to extant species. It also seems likely that nonluminous variants of luminous species can arise frequently through loss of one of more the core genes of the *lux* operon, *luxCDABE* (e.g., Wollenberg et al. 2011). The scattered incidence of *lux* genes in *Vibrionaceae* presumably relates to different ecologies of the different species. It is not clear, however, how having and expressing *lux* genes contributes to the lifestyle of most luminous bacteria, because there are no obvious ecological differences between luminous and nonluminous species except in the case of those species that are bioluminescent symbionts of fish and squids.

Habitats and Ecology of Luminous Bacteria

The luminous *Aliivibrio*, *Photobacterium*, *Vibrio*, and *Shewanella* species occur in the marine environment, whereas *Photorhabdus* species are terrestrial. *Vibrio cholerae* also occurs in brackish environments and freshwater, although strains of this species also commonly occur in coastal seawater (e.g., Kaeding et al. 2007; Urbanczyk et al. 2008).

Marine

Luminous bacteria are globally distributed in the marine environment (*Table 13.1*) and have been isolated from seawater, sediment, and suspended particulates from a wide variety of locations (Baumann and Baumann 1981; Harvey 1952; Zobell 1946). They also commonly colonize marine animals as saprophytes, commensal enteric symbionts, and parasites (Baumann and Baumann 1981; Harvey 1952; Kozukue 1952; Makemson et al. 1997; Makemson and Hermosa 1999; Meighen and Dunlap 1993; Ruby and Morin 1979; ZoBell 1946). They can also be isolated from inanimate surfaces and macroalgae (Makemson et al. 1992). A few species of luminous bacteria establish bioluminescent symbiosis with marine fish and squids (Dunlap 2009; Dunlap et al. 2007; Hastings and Nealson 1981; Haygood 1993; Ruby 1996; Ruby and Morin 1978; Visick and Ruby 2006; Urbanczyk et al. 2011a, b). In seawater, the incidence of luminous bacteria generally is low (from 0.01–40 cells per ml; Nealson and Hastings 1992), with higher numbers in coastal seawater and lower numbers in open ocean and deeper waters (Ruby and Nealson 1978; Ruby et al. 1980). Possibly reflecting this variation, metagenomic analyses of different marine waters have identified the presence of genes related to *luxA* (Martín Cuadrado et al. 2007) and conversely showed an absence of bacterial *lux* genes (Nealson and Venter 2007). Therefore, the geographic distribution of luminous bacteria in the plankton varies substantially.

In contrast to their generally low incidence in seawater, luminous bacteria can attain very high numbers in saprophytic, commensal, parasitic, and symbiotic associations with animals (up to 10^{11} cells per ml in symbiotic habitats; Ruby and Nealson 1976; Dunlap 1984; Nealson and Hastings 1992; Visick and Ruby 2006). For example, luminous bacteria can be readily isolated by enrichment from the muscle tissue and skin of marine fish (e.g., Budsberg et al. 2003; Ast and Dunlap 2005) (*Fig. 13.5*), and *Photobacterium iliopiscarium*, a nonluminous species closely

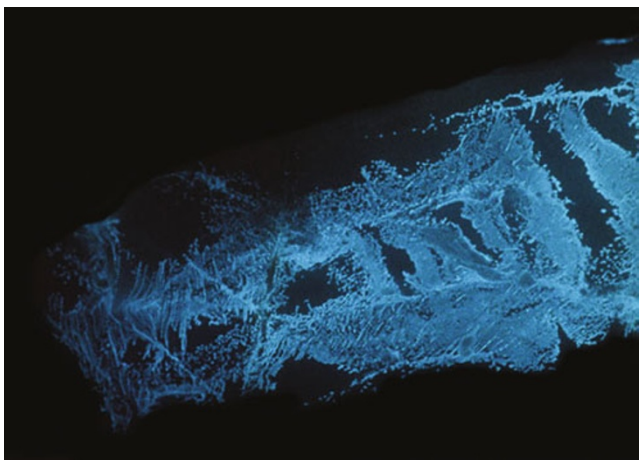

Fig. 13.5

Saprophytic growth of luminous bacteria. **Luminous bacteria have colonized this slice of fish meat, which was photographed in the dark by the light the bacteria produce. Growth of luminous bacteria in and on surfaces of animal tissues is common in nature. This attribute is one means by which luminous bacteria from the environment can be enriched for and isolated**

related to *P. phosphoreum* and *P. kishitanii*, has been isolated from the intestines of several species of cold-water fish and from spoiled packaged fish (Ast and Dunlap 2005; Flodgaard et al. 2005; Onarheim et al. 1994; Urakawa et al. 1999). Saprophytic, commensal, parasitic, and symbiotic habitats have the potential to make substantial contributions to the density and distribution of luminous bacteria in seawater, sediments, and marine snow (Reichelt et al. 1977; O'Brien and Sizemore 1979; Ruby and Morin 1979; Haygood et al. 1984; Nealson et al. 1984; Ramesh et al. 1987; Ruby and Lee 1998; Visick and Ruby 2006), which in turn presumably serve as environmental sources of these bacteria for recolonization of animals. As commensal enteric symbionts of fish, luminous bacteria may contribute significantly to the digestion of crustacean prey through the activity of chitinase (Spencer 1961; Baumann and Schubert 1984; Ramesh and Venugopalan 1989). It should be noted that luminous bacteria coexist with and presumably carry out metabolic activities similar to nonluminous bacteria in these different habitats. Luminous bacteria in general, however, show little specificity when forming opportunistic saprophytic and enteric associations with marine animals such as mussels and clams. This lack of specificity can be attributed to the steady influx of bacteria from the water column, which presumably would prevent selection for specialization (Preheim et al. 2011). The exception to this general lack of specificity is bioluminescent symbiosis, in which the luminous bacteria able to colonize this kind of habitat typically are present as single species.

In contrast to their associations with marine animals, luminous bacteria apparently do not commonly colonize the surfaces of marine algae. Agar digestion is often observed among nonluminous *Vibrio* species and other marine bacteria (e.g., Humm 1946), and various attempts, successful (Makemson et al. 1992) and otherwise, have been made to isolate light-emitting bacteria from algal surfaces. To date, however, only one luminous strain, provisionally identified as a member of *V. harveyi*, that has the ability to digest agar has been isolated from algae (Fukasawa et al. 1987). The uniqueness suggests that the single known isolate of agar-digesting luminous bacteria might have acquired either the genes for agar digestion or the *lux* genes by horizontal gene transfer.

The distributions and numbers of individual species of luminous bacteria tend to correlate with certain environmental factors (Baumann and Baumann 1981). Primary among these factors are temperature and depth (Ruby and Nealson 1978; Yetinson and Shilo 1979; Ruby et al. 1980; Ramaiah and Chandramohan 1987), salinity (Yetinson and Shilo 1979; Feldman and Buck 1984), and nutrient limitation and sensitivity to photooxidation (Shilo and Yetinson 1980; Makemson and Hastings 1982; Haygood and Nealson 1985a). Temperature, along with being an important environmental factor, can influence whether luminous bacteria from environmental samples are detected. For example, *Shewanella hanedai*, which is psychrotrophic, grows and produces light at low temperature (e.g., 4–15 °C) and grows but does not produce light at room temperature (24 °C). Therefore, incubation of platings of environmental samples at lower temperatures may reveal the presence of other luminous species with naturally temperature-sensitive luminescence systems. Temperature relationships would appear to be species-specific, however. For example, *S. woodyi* (found in squid ink and seawater in the Alboran Sea near Gibraltar; Makemson et al. 1997), a species closely related to *S. hanedai*, grows and produces light at room temperature.

Studies of the distribution and density of luminous bacteria in the marine environment traditionally have used visual detection of luminescent colonies arising from seawater spread on nutrient-containing agar plates to identify the presence of these bacteria. However, there are several kinds of luminous bacteria that can be missed with this method. One kind is bacteria that are physiologically cryptic for luminescence, producing visible light in culture only in response to the addition of inducers or other substances to the growth medium (Boettcher and Ruby 1990; Fidopiastis et al. 1999; Nelson et al. 2007) or that require growth at lower than typical room temperatures for light production. Another kind is bacteria with incomplete or defective *lux* operons (O'Grady and Wimpee 2008). Furthermore, enzyme assay and antibody methods have detected luciferase in several *Vibrio* spp. that do not produce visible light in culture (Nealson and Walton 1978; Makemson and Hastings 1986; Kou and Makemson 1988). Similarly, *luxA*-based DNA probes and PCR amplification of *lux* gene sequences have identified *lux* gene-containing bacteria from seawater that do not produce light in culture (Potrikus et al. 1984; Palmer and Colwell 1991; Lee and Ruby 1992; Wimpee et al. 1991; Ramaiah et al. 2000; Grim et al. 2008). These studies demonstrate that bacteria carrying the *lux* genes are more abundant in the marine environment and more phylogenetically diverse than is revealed by analysis of strains isolated on the basis of the production of readily visible levels of light. A counterpoint to this view, however, is the apparently low incidence of *lux* gene sequences in metagenomic databases (Martín Cuadrado et al. 2007; Nealson and Venter 2007), which suggests that luminous *Photobacterium*, *Vibrio*, and *Aliivibrio*, and presumably nonluminous members of these genera as well, represent a very small fraction of the microscopic plankton.

Freshwater

Luminous strains of *V. cholerae* can be isolated from freshwater and brackish estuarine waters (Desmarchelier and Reichelt 1981; West and Lee 1982; West et al. 1983; Palmer and Colwell 1991; Ramaiah et al. 2000; ❯ *Table 13.1*), as well as from coastal seawater (e.g., Urbanczyk et al. 2008). The first such strain, isolated in 1893 by F. Kutscher from the Elbe River in Germany (Harvey 1952), was named "*Vibrio albensis*" and later was synonymized with *V. cholerae* (Reichelt et al. 1976). This species also infects freshwater crustaceans; Thulis and Bernard in 1786 described the luminescence of a freshwater crustacean (possibly the common amphipod *Gammarus pulex*, which apparently was infected with luminous bacteria) from a river in southern France (Harvey 1957). Yasaki (1927) reported the isolation of luminous bacteria from strongly luminous specimens of the freshwater

shrimp, *Xiphocaridina compressa*, in Lake Suwa, Japan. Initially characterized as *Microspira phosphoreum*, the bacterium was later redescribed as *Vibrio yasakii* (Majima 1931). A bacterium responsible for this "light disease of shrimp" was isolated more recently from freshwater shrimp in Lake Biwa, Japan, and identified as non-O1 *V. cholerae* (Shimada et al. 1995). In addition to *V. cholerae* in freshwater habitats, strains of *P. phosphoreum* have been isolated from migrating salmon in the Yukon River, Alaska (Budsberg et al. 2003; Ast and Dunlap 2005); presumably, their association with fish slime protected these marine bacteria from osmotic lysis.

Terrestrial

Luminous bacteria in the terrestrial environment have been noticed mostly as parasites of insects that cause the infected animal to luminesce. Observations of luminous midges, caterpillars, mole crickets, mayflies, and ants, among other infected insects, have been reported from the 1700s into modern times (Harvey 1952; Haneda 1950). As described and summarized by Harvey (Harvey 1952; Harvey 1957), other early reports of terrestrial luminescence attributable to luminous bacteria include luminous mutton, veal, eggs of chickens and lizards, human corpses, and battlefield wounds. Many, and perhaps all, of the observations of luminous insects result from colonization by members of the genus *Photorhabdus*, of which three species are currently described, *Ph. luminescens*, *Ph. temperata*, and *Ph. asymbiotic*a (Fischer–Le Saux et al. 1999; *Table 13.1*). Two of the three *Photorhabdus* species occur as the mutualistic symbionts of soil nematodes of the family Heterorhabditidae (*Table 13.1*) (Akhurst and Dunphy 1993; Forst and Nealson 1996; Forst et al. 1997; Gerrard et al. 2006; Kuwata et al. 2008; Waterfield et al. 2009). They are carried in the intestine of the infective juvenile stage of the nematode and participate in a lethal infection of insect larvae. When the nematode enters the insect, via the digestive tract or other openings, and penetrates the insect's hemocoel, the bacteria are released into the hemolymph, where they use its constituents for growth. The bacteria elaborate a variety of extracellular enzymes that presumably break down macromolecules of the hemolymph. Proliferation of the bacteria leads to death of the insect, and its carcass becomes luminous. The bacteria also produce various extracellular and cell surface-associated factors pathogenic for the insect, as well as bacteriocins and hydroxystilbene and anthraquinone antibiotics, which apparently inhibit the growth of other microorganisms in the insect cadaver and combat scavenging organisms, such as nematodes and amoeba (Akhurst 1982; Sicard et al. 2007; Waterfield et al. 2009). Crystalline protein inclusion bodies of unknown function are also produced (Bintrim and Ensign 1998). The nematodes feed on the bacteria or products of bacterial degradation of the hemolymph enabling them to develop and sexually reproduce (Boemare et al. 1997; Forst et al. 1997). Completion of the nematode life cycle involves reassociation with the bacteria and the emergence from the insect cadaver of the nonfeeding infective juveniles, carrying the bacteria in their intestines. Cells of *Ph. luminescens* presumably are present in soil, but association with the nematode apparently is important for their survival and dissemination. Luminescence of the infected insect larva might function to attract nocturnally active animals to feed on the glowing carcass, thereby increasing the opportunities for the bacterium and the nematode to be disseminated. However, luminescence in *Ph. luminescens*, which is stimulated in laboratory culture by exogenous aldehyde, is not required for successful symbiosis with the nematode; not all strains of *Ph. luminescens* produce luminescence (Akhurst and Boemare 1986; Forst and Nealson 1996; Schmidt et al. 1989). Furthermore, bacteria in the genus *Xenorhabdus*, which are symbiotic with entomopathogenic nematodes in the family Steinernematidae, are ecologically very similar to *Photorhabdus*, except that they do not produce light (Akhurst and Dunphy 1993). The similarities between the lifestyles and activities of *Photorhabdus* and *Xenorhabdus* are postulated to be a case of ecological convergence (Forst and Nealson 1996).

Human clinical infections have yielded *P. asymbiotica*, introduced apparently by spider and insect bites (Farmer et al. 1989; Peel et al. 1999). Luminous battlefield wounds are intriguing in this regard because luminescence apparently was a sign that the wound would heal well (Harvey 1957). Indeed, luminous bacteria will grow and produce light on living mammalian tissue (Johnson 1988). Perhaps antibiotic-producing, nonpathogenic *Photorhabdus* strains promoted wound healing by preventing the growth of putrefying, pathogenic bacteria. On the other hand, the human pathogenicity of *P. asymbiotica* suggests that this species might have killed rather than healed if introduced into wounds. The recent description of *P. asymbiotica* and *P. temperata* and the presence of genetically distinct subspecies within *Ph. luminescens* and *P. temperata* (Fischer Le Saux et al. 1999; Tailliez et al. 2010) indicate that additional diversity, possibly at the species level, may exist in this genus.

Along with terrestrial *Photorhabdus* species, marine luminous bacteria might have been responsible for some of the early reports of luminous meats and eggs, especially if brine was used in their preparation or they otherwise were exposed to seawater. Haneda (1950), following the observation by Molisch (1925) of luminous bacteria growing on beef, demonstrated that luminous bacteria could be isolated from certain samples of beef, pork, and chicken meat. These meats might have contained enough salt to support the growth of marine species, and Haneda cultured the bacteria in media containing 0.5 % salt. However, whether these bacteria were terrestrial (i.e., *Photorhabdus*), from brackish water (i.e., *V. cholerae*), or marine in origin is not known.

Parasitism of Marine Invertebrates

Most of the commonly encountered marine luminous bacteria are not known to be highly invasive or virulent in animals. Many or perhaps all luminous species, however, can act as

opportunistic pathogens upon entering an animal's body through lesions resulting from injury or stress. First noted in marine animals apparently by Viviani in 1805 (Harvey 1957), infections of marine crustaceans by luminous bacteria are common, causing the infected animal to luminesce (Giard 1889; Giard and Billet 1889; Inman 1926). Luminous bacteria inhabit the gut tract and colonize external surfaces of marine crustaceans (Inman 1926; Baross et al. 1978; O'Brien and Sizemore 1979; Lavilla–Pitogo et al. 1992); many are chitinolytic (Spencer 1961; Baumann and Schubert 1984). The bacteria enter the hemocoel of the animal through lesions in the gut or carapace, developing luminescence and killing the animal within a few days. The species of luminous bacteria infecting isopods and amphipods commonly encountered in coastal environments have not been identified in recent times, but they exhibit characters consistent with members of the genera *Aliivibrio*, *Photobacterium*, and *Vibrio* (Hastings and Nealson 1981; P. Dunlap, unpubl. data). Nonluminous bacteria undoubtedly cause similar infections that go unnoticed due to the lack of light production.

As opportunistic pathogens of marine crustaceans, luminous bacteria and their nonluminous relatives have had a profoundly deleterious effect on commercial prawn mariculture (Owens and Busico–Salcedo 2006; Haldar et al. 2011). The development of intensive monoculture of *Penaeus monodon*, the giant tiger prawn, and other penaeids during the 1980s led to a dramatic increase in disease and death of the animals due to luminous bacteria. Shrimp hatchery rearing ponds can become heavily infested with luminous bacteria, with shrimp larvae developing "luminescent vibriosis," a pathogenic state responsible for massive mortalities. The problem continues in grow-out ponds, where the infection localizes to the hepatopancreas in juveniles, limiting the growth of the animals and further increasing losses to mortality (Lavilla–Pitogo and de la Peña 1998). Primarily responsible are strains of *V. harveyi*, though other luminous and nonluminous *Vibrio* species have been identified (Lavilla–Pitogo et al. 1990; Karunasagar et al. 1994; Lavilla–Pitogo and de la Peña 1998; Suwanto et al. 1998; Leano et al. 1998; Austin and Zhang 2006).

Parasitism of Vertebrates

In contrast to the situation with marine invertebrates, luminous bacteria apparently only rarely infect vertebrate animals. The ability of *P. asymbiotica* to infect humans has been mentioned above. *Vibrio harveyi* has been identified in fish disease, and recently, *A. salmonicida* (a pathogen of salmonids and cod) has been shown to contain a *lux* operon (Nelson et al. 2007). Clinical strains of *V. vulnificus* and *V. cholerae* typically are nonluminous, but a luminous strain of *V. vulnificus* has been isolated from a lethal human infection (Oliver et al. 1986; Kaeding et al. 2007), and luminous strains of *V. cholerae* have been isolated from humans suffering from cholera (Jermoljewa 1926). Furthermore, Weleminsky (1895) demonstrated that a nonluminous clinical isolate of *V. cholerae* developed luminescence apparently by passage through pigeon's blood (Harvey 1952). *Vibrio cholerae* strains that are luminous or that contain the *luxA* gene are present in relatively high percentages in freshwater and estuarine environments (West and Lee 1982; West et al. 1983; Palmer and Colwell 1991; Ramaiah et al. 2000). However, O1 or O139 serotypes of *V. cholerae*, which are responsible for life-threatening cases of human diarrheal disease, do not include the light-producing or *luxA* gene–containing strains (Palmer and Colwell 1991; Ramaiah et al. 2000; Grim et al. 2008).

Bioluminescent Symbiosis

A special attribute of a few of the luminous bacteria is the ability to form highly specific, luminescence-based mutualisms, called bioluminescent symbiosis, with certain marine fish and squids (*Table 13.2*). Early work is reviewed in detail by Harvey (1952), Buchner (1965), Herring and Morin (1978), and Hastings and Nealson (1981). In these associations, the animal cultures a dense population of luminous bacteria in a tissue complex called a light organ, providing them with nutrients and oxygen for reproduction and light production. The animal in turn uses the bacterial light for luminescence displays associated with sex-specific signaling, predator avoidance, seeing and attracting prey, or schooling. In most of the bacterially bioluminescent fish, the light organs are associated with the gastrointestinal tract; in others, they are subocular (anomalopids), mandibular (monocentrids), or escal (ceratioids). In squids, the bacterial light organs are found as bilobed structures within the mantle cavity, associated with the ink sac. Accessory tissues associated with the light organ, that is, shutter, lens, and reflector, direct and focus the light the bacteria produce. The light organs open to the external environment, either directly or via the intestinal tract or mantle cavity, allowing the excess bacterial cells to be released from the animal's light organ into the environment as the light-organ population reproduces. In the cases studied, the members of each new host generation of the animal acquire their symbiotic bacteria from the environment. These associations typically are highly specific at the animal family–bacterial species level; members of a family of fish or squid often all harbor the same individual bacterial species as their symbiont (Harvey 1922, 1952; Okada 1926; Harms 1928; Kishitani 1930; Yasaki 1928; Haneda 1938, 1950; Ahrens 1965; Buchner 1965; Hastings 1971; Morin et al. 1975; Herring 1977; Herring and Morin 1978; Nealson 1979; McFall–Ngai 1983; McFall–Ngai and Dunlap 1983; Haygood et al. 1984; Nealson et al. 1984; Dunlap and McFall–Ngai 1987; Wei and Young 1989; McFall Ngai and Morin 1991; McFall Ngai and Ruby 1991; Ruby and Asato 1993; Graf and Ruby 1998; Wada et al. 1999; Woodland et al. 2002; Sasaki et al. 2003; Jones and Nishiguchi 2004; Sparks et al. 2005; Dunlap et al. 2009; Charkrabarty et al. 2011; Dunlap and Nakamura 2011). The bacteria are housed extracellularly, and in most cases they are known to not be obligately dependent on the host for their reproduction, as they colonize a variety of other habitats (Baumann and Baumann 1981; Hastings and Nealson 1981; Visick and Ruby 2006). Bioluminescent symbiosis

Table 13.2

Bacterial species affiliations in bioluminescent symbiosis

Host animal[a]	*Aliivibrio fischeri*	*Aliivibro "thorii"*	*Aliivibrio wodanis*	*Photobacterium leiognathi*	*Photobacterium kishitanii*	*Photobacterium mandapamensis*	*Candidatus* Photodesmus katoptron	Not identified
Squids								
Teuthida								
Loliginidae				+				
Sepiolida								
Sepiolidae	+	+	+					
Fish								
Anguilliformes								
Congridae								+
Argentiniformes								
Opisthoproctidae					+			
Aulopiformes								
Chlorophthalmidae					+			
Gadiformes								
Macrouridae	+				+	+		
Merluciidae						+		
Moridae	+				+			
Lophiiformes								
Centrophrynidae								+
Ceratiidae								+
Diceratiidae								+
Gigantactinidae								+
Himantolophidae								+
Linophrynidae								+
Melanocetidae								+
Oneirodidae								+
Thaumatichtyidae								+

Table 13.2 (continued)

Host animal[a]	*Aliivibrio fischeri*	*Aliivibro "thorii"*	*Aliivibrio wodanis*	*Photobacterium leiognathi*	*Photobacterium kishitanii*	*Photobacterium mandapamensis*	*Candidatus* Photodesmus katoptron	Not identified
Beryciformes								
Anomalopidae							+	
Monocentridae	+							
Trachichthyidae					+			
Perciformes								
Acropomatidae				+	+	+		
Apogonidae						+		
Leiognathidae				+		+		

[a]Data are from (Ast and Dunlap 2005; Ast et al. 2009; Castle and Paxton 1984; Dunlap et al. 2004; Dunlap et al. 2007; Fidopiastis et al.1998; Fukasawa and Dunlap 1986; Haygood and Distel 1993; Haygood et al. 1992; Hendry and Dunlap 2011; Kaeding et al. 2007; Nishiguchi 2000; Wada et al. 2006; Wolfe and Haygood 1991; and Dunlap unpubl. data.

appears to be a unique kind of symbiosis; the bacterial metabolic product needed by the host animal is light, used in bioluminescence displays, rather than a bacterially produced nutrient or enzymatic activity needed for host nutrition (Claes and Dunlap 2000).

Luminous bacteria might also form symbioses with pyrosomes and salps; little is known, however, and the topic remains controversial (Harvey 1952; Buchner 1965). Pyrosome zooids bear a pair of simple photophores that contain intracellular bacteroids, but the involvement of the bacteroids in pyrosome luminescence has been both discounted and supported (Galt 1978; Herring 1978; Mackie and Bone 1978; Haygood 1993). Although the bacteroids have not been cultured, the presence of bacterial luciferase in photophores is consistent with a bacterial origin for pyrosome luminescence (Leisman et al. 1980). A similar proposal for luminous myctophid and stomiiform fish, that the luminescence of the fish's photophores is produced by symbiotic luminous bacteria (Foran 1991), however, has been conclusively refuted (Haygood et al. 1994).

The following information focuses primarily, although not exclusively, on bioluminescent symbiosis in fish. Detailed information on the bioluminescent mutualism of *A. fischeri* with the sepiolid squid *Euprymna scolopes* can be found in the chapter by K. Visick (Chap. 20, "*Vibrio fisheri*: Squid Symbiosis," Vol. 1).

Patterns of Host Affiliation

Six species of luminous bacteria form bioluminescent symbioses with fish and squids, *A. fischeri*, *A.* "*thorii*," *A. wodanis*, *P. kishitanii*, *P. leiognathi*, and *P. mandapamensis*. Their currently known host affiliations are listed in *Table 13.2*. There are over 460 species of bacterially luminous marine fish, in 21 families of seven teleost orders, and several species of squid in two families of two cephalopod orders (Dunlap et al. 2007; Herring and Morin 1978; Nelson 2006) (*Fig. 13.6*). The most numerous of these symbiotic bacteria, due to the exceptional abundance of their host animals in the marine environment, are likely to be *P. kishitanii* and *P. leiognathi*. The hosts of *P. kishitanii* are fish of diverse families in deep-sea habitats worldwide, many of which are abundant, and the hosts of *P. leiognathi* are primarily fish of the family Leiognathidae, which are abundant in shallow coastal waters of Southeast Asia and South Asia (Tiews and Caces Borja 1965; Kühlmorgen–Hille 1974; Herring and Morin 1978; Cohen et al. 1990; Orlov and Iwamoto 2006; Dunlap et al. 2007; Dunlap et al. 2009). The bioluminescent symbionts of deep-sea fish previously were thought to be *P. phosphoreum*, but detailed phylogenetic analyses of the *phosphoreum* species group identified *P. kishitanii* as the species occurring in light organs of deep-sea fish. Despite extensive testing, no bonafide member of *P. phosphoreum* has been found in light-organ symbiosis (Ast and Dunlap 2005; Ast et al. 2009; Dunlap and Ast 2005; Dunlap et al. 2007; Kaeding et al. 2007; Urbanczyk et al. 2007). Similarly, strains of bacteria from light organs of the sepiolid squids *Sepiola affinis* and *Sepiola robusta*, previously identified as *A. logei* (*V. logei*) (Fidopiastis et al. 1998), were recently identified based on detailed phylogenetic criteria as three entities, *A. fischeri*; *A.* "*thorii*", a newly recognized bacterial clade; and *A. wodanis*, a previously described species newly recognized as a bioluminescent symbiont; apparently no bonafide member of *A. logei* has been found in light-organ symbiosis (Ast et al. 2009). In addition to these bacteria, strains identified as *V. harveyi* have been found in the light organ of larval leiognathid fish (Dunlap et al. 2008); it is not yet known if *V. harveyi* is present as an incidental, transient colonizer of the nascent light organ of this fish, as a pathogen, or, possibly, as an actual symbiont (Dunlap et al. 2008).

Species Specificity, Cosymbiosis, and Symbiont: Host Codivergence

Previously, bioluminescent symbioses were characterized as species specific, with the light organ of each animal thought to harbor a single, pure culture of bacteria and with the members of each family of fish or squids thought to all harbor the same bacterial species as their symbiont (Hastings and Nealson 1981; Nealson and Hastings 1992; Dunlap and Kita–Tsukamoto 2006). This pattern of specificity still generally holds, but several deviations from a strict host family–bacterial species specificity have been identified. On the one hand, individual light organs of certain squid and fish have been found to harbor two bacterial species, a situation termed cosymbiosis. In contradicting a strict one-to-one relationship, cosymbiosis requires the mechanism by which the host might select its symbiotic bacteria, such as surface recognition, to respond to features common to both bacterial species or to distinct features of each (Ast et al. 2009; Fidopiastis et al. 1998; Dunlap et al. 2007; Dunlap et al. 2008; Kaeding et al. 2007; Dunlap et al., unpubl. data). On the other hand, different host species and genera within a family have been found to harbor different species of bacteria (*Table 13.2*). One example of this breakdown of host family level bacterial specificity is the presence in light organs of *Acropoma hanedai* of *P. kishitanii*, whereas *P. mandapamensis* is present as the primary symbiont in light organs of *Acropoma japonicum*. Another is the presence in different species of *Coelorinchus* of *P. kishitanii* or *A. fischeri* (Dunlap et al. 2007; Kaeding et al. 2007; Wada et al. 2006). These discrepancies suggest that a strict genetically based host selection of a specific symbiotic bacterium (McFall Ngai and Morin 1991) may not be operative in bioluminescent symbiosis or may not be operative for all bacterially luminous animals.

Consistent with this possibility is the lack of codivergence, that is, cospeciation, between host and symbiont lineages (*Fig. 13.7*). Genetic selection might reasonably lead to a codivergence, as reported for squid-symbiotic bacteria (Nishiguchi et al. 1998), but instead of congruent host and symbiont phylogenies, detailed phylogenetic analysis based on homologous genes, suitable numbers of strains, and a diversity of hosts reveals instead that the patterns of symbiont affiliations for fish and squids are strikingly noncongruent

Fig. 13.6

Bacterially luminous fish*. Shown are a few of the more than 460 species of fish that host luminous bacteria as bioluminescent symbionts. Counterclockwise from the top, the fish are *Physiculus japonicus* (Gadiformes: Moridae) (photo by A. Fukui), host of *P. kishitanii* and, less commonly, *A. fischeri*; *Eubleekeria jonesi* (Perciformes: Leiognathidae) (photo by P.V. Dunlap), host of *P. leiognathi*; *Acropoma japonicus* (Perciformes: Acropomatidae) (photo by A. Fukui), host of *P. mandapamensis* and, less commonly, *P. leiognathi*; *Chlorophthalmus nigromarginatus* (Aulopiformes: Chlorophthalmidae), host of *P. kishitanii*; *Monocentris japonicus* (Beryciformes: Monocentridae) (photo by P.V. Dunlap), host of *A. fischeri*; and *Aulotrachichthys prosthemius* (Beryciformes: Trachichthyidae) (photo by A. Fukui), host of *P. kishitanii

(Dunlap et al. 2007). Furthermore, phylogenetically distantly related hosts, for example, bacterially luminous aulopiforms, most gadiforms, and certain beryciforms, all harbor the same bacterial species, *P. kishitanii*, whereas some closely related hosts, such as the acropomatid fish *A. hanedai* and *A. japonicum*, as noted above, harbor distinct species, *P. kishitanii* and *P. mandapamensis*, respectively (Dunlap et al. 2007) (*Fig. 13.7*).

An alternative hypothesis to account for the observed patterns of symbiont–host affiliation in bioluminescent symbiosis is environmental congruence. This hypothesis, first outlined by Hastings and Nealson (1981), links the differing environmental distributions of different species of luminous bacteria, that is, where each species is most abundant, with the environmental distribution of its host animal (Dunlap et al. 2007; Hastings and

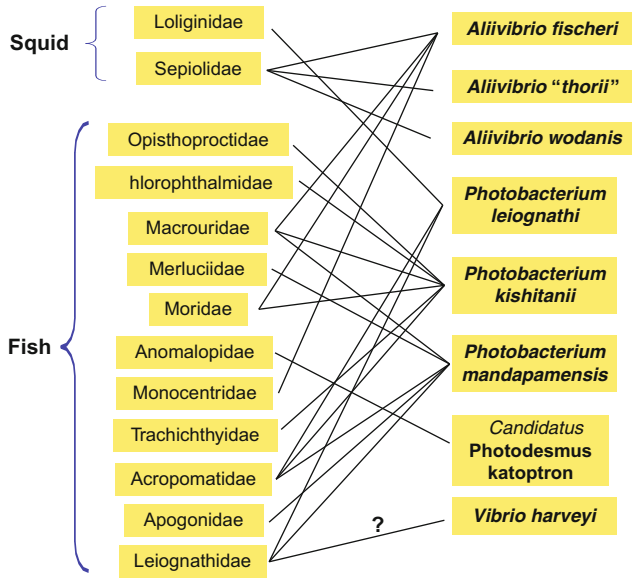

Fig. 13.7

***Host affiliations of symbiotic luminous bacteria*. Families of bacterially luminous squids and fish are listed on the left, with lines to the corresponding bacterial species on the right that have been isolated from light organs of these animals. Different members of individual families of fish and some squids often harbor different species of bacteria, in some cases within the light organ of the same host specimen. Some of the bacteria, for example, *A. fischeri*, *P. kishitanii*, *P. mandapamensis*, are found in light organs of a diversity of fish and squids. These attributes highlight the lack of strict family level bacterial species specificity and the lack of phylogenetic congruence between host and symbiont in bioluminescent symbiosis (Dunlap et al. 2007; Kaeding et al. 2007). The question mark for the link from Leiognathidae to *V. harveyi* reflects the single instance that this bacterial species has been isolated from light organ symbiosis (Dunlap et al. 2008)**

Nealson 1981; Kaeding et al. 2007; Dunlap et al. 2008). Temperature, which influences the presence and relative numbers of the different species of luminous bacteria in the marine environment, may be the key environmental factor; deeper, colder dwelling hosts harbor the more psychrotrophic luminous species found in those habitats, *P. kishitanii*, for example, as their bioluminescent symbiont, whereas shallower and warmer dwelling hosts harbor the more mesophilic luminous species found in those habitats, *A. fischeri* and *P. leiognathi*, for example. An important further consideration is the ontogenetic ecology of the host. Early life history stages of these animals, for example, eggs, larvae, and juveniles, often are distributed in the environment differently from adults. The key factor therefore may be where in the environment the animal is when it is developmentally ready to initiate symbiosis. The luminous bacterial species most abundant in and adapted to the conditions of those habitats presumably would the ones most likely to initiate symbiosis (Dunlap et al. 2007; Kaeding et al. 2007). Information about early life history stages of bacterially luminous animals, especially fish, is very limited, but evidence is beginning to accumulate that supports the environmental congruence hypothesis (Dunlap et al. 2008). Nonetheless, some form of host selection must be occurring because to date only luminous bacteria, and only certain species of luminous bacteria, have been found in light organs of fish and squid. Most likely, a combination of environmental congruence and some level of selection are operative.

The luminous bacteria symbiotic with two other groups of fish, the flashlight fish, family Anomalopidae, and bacterially luminous deep-sea anglerfish, in order Lophiiformes, present a possible contrast to the apparent lack of strict species specificity in bioluminescent symbiosis. Microscopic analysis showing the presence of masses of bacterial cells within the light organs, assays specific for bacterial luciferase, and other studies convincingly demonstrate the bacterial nature of light emission in these fish (Bassot 1966; Harvey 1922; Haygood et al. 1984; Kessel 1977; Leisman et al. 1980; Munk et al. 1998), but the bacteria from light organs of these fish have not been grown in laboratory culture despite numerous attempts (Hastings and Nealson 1981; Haygood 1993; Hendry and Dunlap 2011; Herring and Morin 1978). The inability to grow these bacteria in the laboratory suggests that they have lost the ability to reproduce outside the host light organ and are therefore might be obligately dependent on their hosts (Haygood 1993; Haygood and Distel 1993). An obligate relationship presumably would lead to a very high degree of specificity and possibly also to codivergence between the host fish and its bacteria. 16S rRNA gene sequence analysis of the symbiotic bacteria of two ceratioids, representing different families of lophiiformes, places these bacteria as members of *Vibrionaceae* and possibly as a new bacterial species in each fish (Haygood and Distel 1993; Hendry and Dunlap 2011). Analysis of the *luxA* and 16S rRNA genes of anomalopid symbionts suggested these bacteria are members of *Vibrio* and that different genera of the fish harbor bacteria that differ at greater than the strain level (Haygood 1990; Wolfe and Haygood 1991). Consistent with and extending these findings, a recent detailed multilocus analysis classified the bacteria symbiotic with the anomalopid fish *Anomalops katoptron* as a new *Vibrionaceae* genus and species, *Candidatus* Photodesmus katoptron, which is closely related to *Vibrio* (Hendry and Dunlap 2011).

Symbiont Acquisition

In the few cases studied, bacterially luminous squid and fish have been found to acquire their symbiotic luminous bacteria from the environment with each new host generation. The sepiolid squid *E. scolopes* acquires cells of *A. fischeri* from seawater shortly after hatching from the egg as aposymbiotic juveniles; bacteria other than the native symbiont fail to colonize the light organ or do so less effectively (Wei and Young 1989; McFall Ngai and Ruby 1991). In fish, the symbiotic bacteria are acquired later, following development of larvae and inception of light organ formation (Wada et al. 1999; Dunlap et al. 2008;

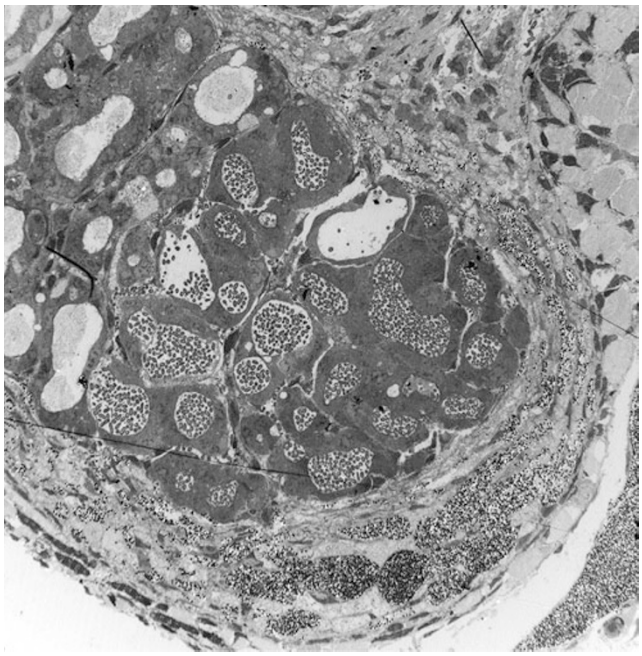

Fig. 13.8
Developing light organ. **This electron micrograph of a section of the light organ of the fish *Nuchequula nuchalis* (Leiognathidae) (micrograph prepared by Sasha Meschinchi, Microscopy and Imaging Laboratory, University of Michigan) shows tubules of the nascent light organ. Some tubules are empty, whereas some are filled or becoming filled with bacteria**

Dunlap et al. 2009; Dunlap et al. 2012) (*Fig. 13.8*), which is consistent with acquisition of the bacteria from the environment. Also consistent with environmental acquisition, symbiotic bacteria apparently are not present on eggs within the ovary of anomalopid fish (Haygood 1993).

Quorum Sensing Control (Autoinduction) of Bacterial Luminescence

In many luminous bacteria, luciferase synthesis and luminescence are regulated in a population density-responsive manner. At low population density, very little luciferase is synthesized, and consequently, little light is produced, whereas at high population density, luciferase levels are induced 100–1,000-fold and light levels increase by 10^3–10^6-fold. Population density–responsive induction of luciferase synthesis and luminescence is controlled in part by the production and accumulation in the cell's local environment of small signal molecules, called autoinducers (acyl-homoserine lactones, AHLs, and other low molecular weight compounds), which function via regulatory proteins to activate transcription of the *lux* operon. Originally called autoinduction and discovered through study of the pattern of luminescence and luciferase production of *V. harveyi* in batch culture (Nealson et al. 1970), this gene regulatory mechanism is now referred to as quorum sensing to reflect its relationship with population density (Fuqua et al. 1994; Greenberg 1997; Hastings and Greenberg 1999; Miller and Bassler 2001).

Over the past 30 years, there has been a very substantial accumulation of information on how two luminous bacteria, *V. harveyi* (a key strain recently was reclassified as *V. campbellii*; Lin et al. 2010) and *A. fischeri*, regulate luminescence by quorum sensing. Studies in these two bacteria established a base of knowledge that led to the discovery of biochemically and genetically homologous quorum sensing systems in a wide variety of nonluminous bacteria; quorum sensing controls many cellular activities other than light production, particularly the production of extracellular enzymes and other extracellular factors thought to be adaptive for bacteria at high population density and in association with animal and plant hosts (Fuqua et al. 1996; Dunlap 1997; Bassler, et al. 1997; Swift et al. 1999; Callahan and Dunlap 2000; Waters and Bassler 2005; Dunlap and Kita–Tuskamoto 2006; Higgins et al. 2007). Quorum sensing therefore is not only not unique or even special to luminous bacteria, it is also widespread and evolutionarily conserved across a diversity of bacteria. As a signal–response mechanism by which bacteria can assess their local population density, quorum sensing might have arisen evolutionarily as a diffusion sensor or efficiency sensor (Redfield 2002; Hense et al. 2007), mediating whether or not cells produce extracellular enzymes and other factors for obtaining nutrients. Substantial attention has been placed recently on the definition and correct usage of terms for quorum sensing and other chemically mediated bacterial interactions (Platt and Fuqua 2010; Stacy et al. 2012). This form of genetic regulation has been studied in detail in *A. fischeri* and *V. harveyi* but remains poorly understood in other luminous bacteria (Meighen 1999). It should be noted also that some luminous bacteria express luminescence constitutively during growth in batch culture (Katznelson and Ulitzur 1977; P. Dunlap, unpubl. data) and therefore apparently do not use a quorum sensing system to control luminescence. An overview of quorum sensing in *V. harveyi* and *A. fischeri* is provided here, and more information on this topic is provided in the chapters by B. Bassler (Chap. 22, "Quorum Sensing," Vol. 2) and K. Visick (Chap. 20, "*Vibrio fisheri*: Squid Symbiosis," Vol. 1).

Early Studies of Quorum Sensing Control of Luminescence. In *A. fischeri* and *V. harveyi*, expression of the *lux* operon, which initially is low in early exponential phase cultures, induces strongly as cultures attain the high cell densities associated with late exponential to early stationary phases of growth (Hastings and Greenberg 1999). Early analyses of the "phases of luminescence" in culture (e.g., Baylor 1949; Farghaly 1950) were followed by the demonstration that luciferase synthesis is inducible and that complete medium contained a compound inhibitory to induction (Nealson et al. 1970; Eberhard 1972). During growth, cells of *A. fischeri* and *V. harveyi* were found also to release into the medium species-specific positively acting secondary metabolites, called autoinducers. These compounds accumulate in the growth medium in a population density-dependent manner, and once they attain threshold concentrations, they induce luciferase synthesis (Nealson et al. 1970;

Eberhard 1972; Nealson 1977; Nealson and Hastings 1979; Ulitzur and Hastings 1979; Rosson and Nealson 1981).

Analysis of quorum sensing attained benchmarks of progress in the 1980s with the identification of autoinducer signal molecules and *lux* regulatory genes. The first autoinducer, 3-oxo-hexanoyl-HSL (3-oxo-C6-HSL), and the first *lux* regulatory genes, *luxI* (encoding 3-oxo-C6-HSL synthase; Schaefer et al. 1996) and $luxR_{Af}$ (encoding acyl-HSL receptor/transcriptional activator), were identified in *A. fischeri* (Eberhard et al. 1981; Engebrecht et al. 1983; Engebrecht and Silverman 1984), followed by identification of 3-hydroxybutanoyl-HSL and a nonhomologous regulatory gene, $luxR_{Vh}$, in *V. harveyi* (Cao and Meighen 1989; Martin et al. 1989; Showalter et al. 1990).

Quorum Sensing Regulatory Circuitry. Ongoing progress since the 1980s has substantially deepened understanding of the quorum sensing genetic regulatory circuitry controlling luminescence in *V. harveyi* and *A. fischeri*. The two regulatory systems are strikingly different. In *V. campbellii* (previously classified as *V. harveyi*; Lin et al. 2010), three chemically distinct autoinducers are produced, 3-hydroxybutanoyl-HSL (harveyi autoinducer-1, HAI-1), (2 S,4 S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate (*V. harveyi* autoinducer-2, $AI\text{-}2_{Vh}$), and (S)-3-hydroxytridecan-4-one (cholerae autoinducer, CAI-1) (Cao and Meighen 1989; Cao and Meighen 1993; Chen et al. 2002; Higgins et al. 2007) (*Fig. 13.9*). Synthesis of HAI-1 is dependent on LuxM (Bassler et al. 1993), synthesis of $AI\text{-}2_{Vh}$ is catalyzed by LuxS (Schauder et al. 2001), and synthesis of CAI-1 is catalyzed by CqsA (Kelly et al. 2009; Wei et al. 2011). Each of these molecules is specifically recognized by a different cytoplasmic membrane-associated two-component histidine kinase receptor, LuxN (Bassler et al. 1993; Freeman et al. 2000), LuxPQ (Bassler et al. 1994b), and CqsS (Henke and Bassler 2004), respectively (*Fig. 13.9*). When concentrations of the autoinducers are low, such as at low

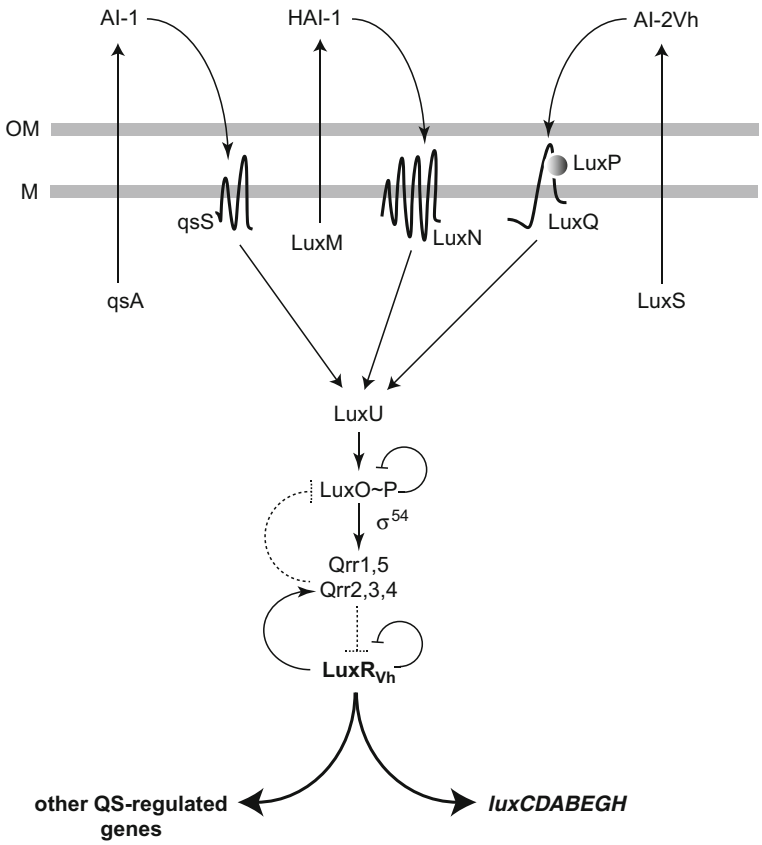

Fig.13.9

Regulatory circuitry controlling luminescence in V. campbellii. **The expression of *lux* operon, and of other quorum sensing-regulated genes, in *V. campbellii* (previously classified as *V. harveyi*), is coordinated by three chemically distinct autoinducers, HAI-1, $AI\text{-}2_{Vh}$, and CAI-1, that modulate the phosphorylation state of *luxU*. The synthesis of each autoinducer is catalyzed by a different protein, LuxM, LuxS, and CqsA, and each is recognized by a different cytoplasmic membrane-associated two-component histidine kinase receptor, LuxN, LuxPQ, and CqsS, respectively. Low concentrations of the autoinducers lead to phosphorylation of LuxO and via quorum regulatory RNAs to the destabilization the $luxR_{Vh}$ transcript, thereby blocking *lux* operon transcriptional activation by $LuxR_{Vh}$. High concentrations of the autoinducers reverse the phosphorylation cascade, allowing formation of $LuxR_{Vh}$ and activation of *lux* operon transcription. Arrows indicate positive interactions or transcriptional activation, whereas bars indicate blocking of transcription. See the text for details and references (Redrawn from Tu et al. (2010))**

population density or in habitats in which the autoinducers diffuse away rapidly from cells, that is, seawater, the receptor proteins function as kinases, transferring phosphate to LuxU, a histidine-phosphotransfer protein. LuxU then transfers the phosphate to LuxO, a DNA binding response regulator, the expression of which is subject to repression by LuxT (Bassler 1999; Bassler et al. 1994a; Cao et al. 1995; Freeman and Bassler 1999a, b; Surete et al. 1999; Lilley and Bassler 2000; Lin et al. 2000; Miyamoto et al. 2003; Waters and Bassler 2006). LuxO ~ P, together with sigma factor σ^{54}, then activates expression of genes coding for five small quorum regulatory RNAs (Qrr), Qrr1 through Qrr5 (Lenz et al. 2004; Tu and Bassler 2007). The Qrr RNAs bind and destabilize the *luxR*$_{Vh}$ transcript, blocking production of LuxR$_{Vh}$ protein, the transcriptional activator of the *lux* operon (Showalter et al. 1990; Swartzman et al. 1992), and thereby preventing activation of *lux* operon transcription. Conversely, once autoinducer concentrations have attained high levels in the cell's local environment, they bind to their receptors, causing the receptors to switch from kinases to phosphatases, leading to the dephosphorylation of LuxO. With the resulting cessation of *qrr* gene transcription, *luxR*$_{Vh}$ message is produced and translated, and LuxR$_{Vh}$ activates *lux* operon transcription. Negative autoregulation of LuxR$_{Vh}$ adds additional complexity to this system (Chatterjee et al. 1996; Miyamoto et al. 1996), as does the negative autoregulation of LuxO and posttranscriptional control of LuxO by Qrr sRNAs (Tu et al. 2010; ❯ *Fig. 13.9*). The complexity of this regulatory system apparently benefits *V. campbellii* by allowing a fine tuning of its quorum sensing response to differences in the various habitats the bacterium colonizes (Waters and Bassler 2005; Ng and Bassler 2009; Tu et al. 2010).

Quorum sensing control of luminescence in *A. fischeri* involves a very different regulatory system. A population density-dependent accumulation of the autoinducer 3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL), a membrane-permeant molecule, triggers induction when it reaches a critical concentration (❯ *Fig. 13.10*). Synthesis of 3-oxo-C6-HSL is catalyzed by LuxI, an acyl-homoserine lactone synthase. The regulatory genes, *luxR*$_{Af}$ and *luxI*, are directly linked to the *lux* operon (❯ *Figs. 13.2*, ❯ *13.10*). The *luxR*$_{Af}$ gene, which is upstream of the *lux* operon and divergently transcribed from it, encodes a transcriptional activator protein, LuxR$_{Af}$, which associates with 3-oxo-C6-HSL, forming a complex that binds at a site in the *lux* operon promoter and that facilitates the binding of RNA polymerase, thereby activating transcription of the genes for light production, *luxICDABEG*. Because *luxI* is a gene of the *lux* operon, increased transcription leads to increased synthesis of 3-oxo-C6-HSL in an autocatalytic, positive feedback manner.

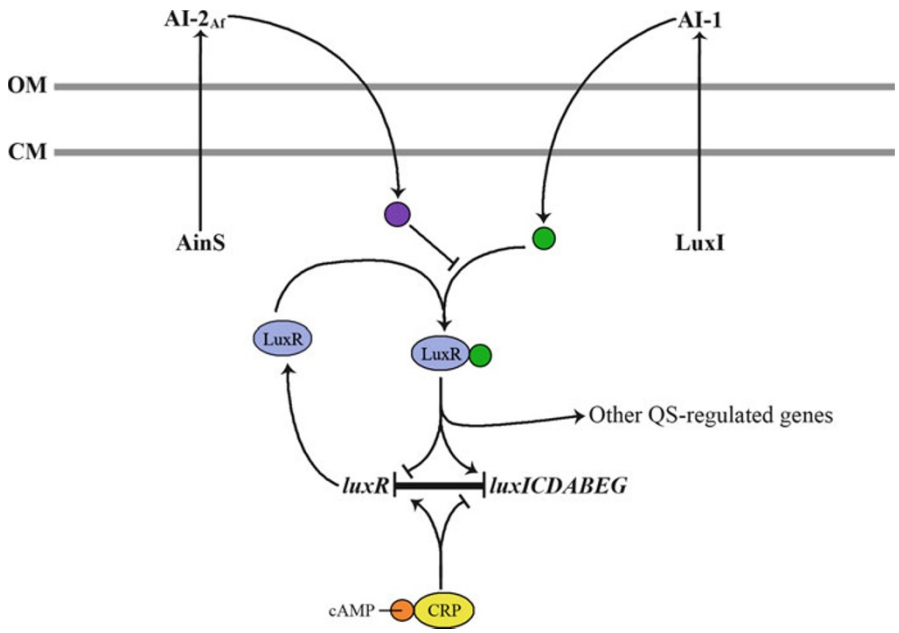

Fig. 13.10

***Regulatory circuitry controlling luminescence in A. fischeri*. The expression of the *lux* operon, and of other quorum sensing-regulated genes, in *A. fischeri* is mediated primarily by the concentration of AI-1, which forms a complex with LuxR$_{Af}$. Synthesis of AI-1 is dependent on LuxI, and the AI-1/LuxR$_{Af}$ complex activates *luxICDABEG* transcription. Together with cAMP, the CRP protein activates expression from the *luxR*$_{Af}$ promoter, increasing synthesis of LuxR$_{Af}$ and potentiating the system to induce strongly once sufficient AI-1 has accumulated. Increased expression from the *lux* operon promoter leads to a stimulation of AI-1 synthesis in an autocatalytic, positive feedback manner, resulting in a rapid and strong induction of luciferase synthesis. A second autoinducer, AI-2$_{Af}$, interacts with LuxR$_{Af}$, interfering with the interaction between AI-1 and LuxR$_{Af}$. The hypothesized AI-2$_{Af}$/LuxR$_{Af}$ complex is thought to be transcriptionally less effective and functions to delay the onset of AI1/LuxR$_{Af}$ activation of *luxICDABEG* transcription. See the text for details and references.**
Figure provided by K. Dougan, University of Michigan

The result is a rapid and strong induction of luciferase synthesis and luminescence (Engebrecht et al. 1983; Engebrecht and Silverman 1984; Kaplan and Greenberg 1985; Eberhard et al. 1991; Schaefer et al. 1996; Stevens and Greenberg 1997).

Other regulatory factors modulate quorum sensing in *A. fischeri*. GroEL is necessary for production of active $LuxR_{Af}$ (Adar et al. 1992; Adar and Ulitzur 1993; Dolan and Greenberg 1992), and 3' 5'-cyclic AMP (cAMP) and cAMP receptor protein (CRP) activate transcription of *luxR* and thereby potentiate the cell's response to 3-oxo-C6-HSL (Dunlap and Greenberg 1985; 1988; Dunlap 1989; Dunlap and Kuo 1992). $LuxR_{Af}$/3-oxo-C6-HSL also negatively autoregulates $luxR_{Af}$ expression (Dunlap and Greenberg 1988; Dunlap and Ray 1989), and a second autoinducer, octanoyl-HSL, synthesis of which is catalyzed by AinS, interacts with $LuxR_{Af}$ apparently to delay *lux* operon induction (Eberhard et al. 1986; Kuo et al. 1996; Hanzelka et al. 1999) (◗ *Fig. 13.10*). Under anaerobic conditions, Fnr contributes to *lux* operon expression (Müller Breitkreutz and Winkler 1993). A homolog of the *V. harveyi luxO* gene is carried by *A. fischeri*, and as with *V. harveyi*, LuxO in *A. fischeri* functions as a repressor of luminescence (Miyamoto et al. 2000, 2003), apparently in a *qrr*-dependent manner (Miyashiro et al. 2010). In addition, LitR, which has substantial sequence similarity to $LuxR_{Af}$, positively regulates *lux* operon expression (Fidopiastis et al. 2002), and LexA is thought to contribute to control of luminescence (Shadel et al. 1990; Ulitzur and Dunlap 1995).

Despite the many differences in the quorum sensing regulatory systems of *V. harveyi* and *A. fischeri*, there are some commonalities. The C-terminal half of the *A. fischeri* AinS protein is 34 % identical to the *V. harveyi* LuxM protein, and the N-terminal portion of *A. fischeri* AinR, encoded by *ainR*, a gene downstream of *ainS*, is 38 % identical to the amino terminus of *V. harveyi* LuxN (Gilson et al. 1995). Whether AinR itself, possibly through interaction with C8-HSL, plays a role in *lux* regulation in *A. fischeri* (Gilson et al. 1995; Kuo et al. 1994) has not been established. The deduced amino acid residue sequence of *A. fischeri* LuxO is approximately 70 % identical to that of *V. harveyi* (Miyamoto et al. 2000), and a gene immediately downstream of *luxO* in *A. fischeri* is likely to be a homolog of *V. harveyi luxU*. Whether these homologies indicate overlaps in quorum sensing control, however, remains to be determined.

Another commonality between the two systems is expression of the *lux* operons of both species is dependent on cyclic AMP (cAMP) and cAMP receptor protein (CRP) (Ulitzur and Yashphe 1975; Chen et al. 1985; Dunlap and Greenberg 1985; 1988; Dunlap 1989; Dunlap and Kuo 1992). Consistent with this dependence, the regulatory regions upstream of the *lux* operons of both species contain a CRP binding site (Engebrecht and Silverman 1987; Devine et al. 1988; Miyamoto et al. 1988). Mutants of *V. harveyi* and *A. fischeri* apparently defective in adenylate cyclase and unable to produce light in the absence of added cAMP have been isolated and characterized (Ulitzur and Yashphe 1975; Dunlap 1989). Furthermore, CRP from *V. harveyi* has been purified and shown to be immunologically and functionally homologous to CRP of *Escherichia coli* (Chen et al. 1985), and the *cya* and *crp* genes of *A. fischeri* have been cloned and found to be highly similar in deduced amino acid residue sequence to *E. coli cya* and *crp* genes (P. Dunlap et al., unpubl. data). In *V. harveyi*, cAMP-dependent binding of CRP activates *lux* operon expression as well as expression of $luxR_{Vh}$ (Chatterjee et al. 2002). Studies with *A. fischeri* and with *E. coli* carrying the *A. fischeri luxR–luxICDABEG* genes indicate that a major effect of cAMP–CRP is to activate expression of $LuxR_{Af}$ while repressing transcription from the *lux* operon promoter (Dunlap and Greenberg 1985; 1988; Dunlap and Kuo 1992; Shadel et al. 1990), although other important *lux* regulatory effects have also been described (Shadel and Baldwin 1991; 1992a, b). Control by cAMP–CRP suggests that the luminescence systems of these bacteria might be part of the cellular response to stresses associated with nutrient limitation and decreasing growth rate.

Physiological Control of Luminescence. The presence of glucose can suppress bacterial luminescence; this catabolite repression effect presumably operates by modulating the levels of cAMP and CRP in the cell (Nealson et al. 1972; Meighen and Dunlap 1993; Dunlap 1997). In *V. harveyi*, catabolite repression by glucose in batch culture is permanent and is reversed by addition of cAMP (Nealson et al. 1972), whereas glucose repression of luminescence in *A. fischeri* is temporary, not reversed by addition of cAMP, and is eliminated by prior growth in the presence of glucose (Ruby and Nealson 1976). Complicating these differences from studies in batch culture are studies of *A. fischeri* grown in phosphate-limited chemostat culture; glucose repression of luminescence under these conditions is permanent, and it is reversible by cAMP (Friedrich and Greenberg 1983). A further complication for studies of cAMP control of luminescence in *A. fischeri* is the presence in this species of a novel, exceptionally potent periplasmic cyclic nucleotide phosphodiesterase specific for extracellular 3' 5'-cyclic nucleotides; activity of the enzyme enables cells to grow on exogenously supplied cAMP as a sole source of carbon and energy, nitrogen, and phosphorus (Dunlap et al. 1992; Dunlap and Callahan 1993; Callahan et al. 1995).

In addition to glucose, other physiological factors can strongly influence the amount of light produced by luminous bacteria grown in laboratory culture. Oxygen, amino acids, iron, and osmolarity have distinct effects, depending on the species studied (Harvey 1952; Coffey 1967; Nealson et al. 1970; Hastings and Nealson 1977; Makemson and Hastings 1982; Dunlap 1985; Haygood and Nealson 1985a; Hastings et al. 1987; Guerrero and Makemson 1989; Dunlap 1991). Those factors that stimulate growth rate, such as readily metabolized carbohydrates, tend to decrease light production and luciferase synthesis. They do so presumably by directing the consumption of oxygen and reducing power ($FMNH_2$) away from luciferase (e.g., McElroy and Seliger 1962; Coffey 1967) and by indirectly or directly influencing *lux* gene expression (Dunlap and Greenberg 1985; Dunlap 2000). Conversely, factors that restrict growth rate, such as limitation for iron and low or high osmolarity of the growth medium, depending on the species, tend to stimulate the synthesis and activity of luciferase (Hastings and Nealson 1977;

Makemson and Hastings 1982; Haygood and Nealson 1985; Hastings et al. 1987; Dunlap 1991). The mechanisms by which these factors operate, however, are not well understood, although they presumably interface with the quorum sensing control circuitry in some way.

Other Genes Subject to Quorum Sensing Regulation. Studies of *V. harveyi* led to the first demonstration of non-*lux* quorum-sensing-regulated genes. In *V. harveyi*, the production of the fatty acid storage product poly-β-hydroxybutyrate is controlled in a cell density-dependent manner by 3-OH-C4-HSL (Sun et al. 1994; Miyamoto et al. 1998). Along with that finding, $LuxR_{Vh}$ has been shown to control various non-*lux* genes and to act as either a transcriptional activator or repressor (Chatterjee et al. 1996; Miyamoto et al. 1996; Miyamoto and Meighen 2006; Waters and Bassler 2006; Pompeani et al. 2008). In *A. fischeri*, proteomic analysis of mutants defective in *luxR*, *luxI*, and *ainS* revealed the presence of several quorum-sensing-controlled non-*lux* genes, components of a LuxR-dependent quorum-sensing regulon; these genes code for a variety of different of proteins, some of which apparently contribute to the ability of *A. fischeri* to colonize its squid host, *E. scolopes* (Callahan and Dunlap 2000; Qin et al. 2007). Transcript analysis confirmed and extended these results to several additional genes (Antunes et al. 2007).

Isolation, Cultivation, Storage, and Identification of Luminous Bacteria

When working with luminous bacteria, and particularly when isolating new strains from nature, the possibility that these bacteria could be pathogenic (e.g., Kaeding et al. 2007) should always be kept in mind and appropriate care to avoid infection should always be used. Additional and detailed information on the isolation, cultivation, and phenotypic characterization of luminous bacteria can be found in Nealson (1978), Baumann et al. (1984), Baumann and Baumann (1981), Farmer and Hickman–Brenner (1992), and Baumann and Schubert (1984).

Isolation

Luminous bacteria can be isolated from most marine environments, and two methods, direct plating of seawater and enrichment from marine fish skin, are effective and simple for this purpose. An easily prepared complete medium that is suitable for growing all known luminous bacteria, LSW-70, contains natural or artificial seawater, diluted to 70 % of full strength, 10 g l^{-1} tryptone or peptone, and 5 g l^{-1} yeast extract, with 15 g l^{-1} agar for solid medium (Dunlap et al. 2004). Sugars and sugar alcohols (i.e., glucose, glycerol) are unnecessary for good growth and luminescence and can lead to acid production and death of cultures (Hill 1928; Johnson and Shunk 1936; Dunlap et al. 1995); their use in isolation media should be avoided. For isolations from environments where high numbers of bacteria that form spreading colonies may be present, such as coastal seawater, sediment, and intestinal tracts of marine animals, the use of agar at 4 % (40 g l^{-1}) (Baumann et al. 1984) is recommended. This harder, less moist agar limits the ability of bacteria motile on solid surfaces, for example, certain peritrichously flagellated bacteria and bacteria that move by gliding motility, to spread over the plate and cause cross contamination of colonies.

Direct plating of seawater involves simply spreading an appropriate volume, typically 10–100 µl for coastal seawater, of the sample on one or more plates and incubating at room temperature or, preferably, cooler temperatures, such as 15–20 °C. For open ocean seawater and other samples with a lower number of bacteria, larger volumes, for example, 100 ml to 1 l, can be filtered through membrane filters with a pore size of 0.2 µm or 0.45 µm to collect the bacteria. The filters are then placed, bacteria side up, on plates of the above medium. Once colonies have arisen, usually within 18–24 h at room temperature and longer for lower temperatures, the plates can be examined in a dark room. Luminous colonies can then be picked (sterile wooden toothpicks are suitable for this purpose) and streaked for isolation on fresh plates of the same medium. Use of a red light, such as a photographic darkroom light, on a variable intensity control can make the picking of luminous colonies easier; by adjusting the red light, colonies of nonluminous bacteria can be made to appear reddish, whereas luminous colonies are blue due to their luminescence. Samples collected from warm waters and incubated at room temperature are more likely to yield *V. harveyi* and related luminous *Vibrio* species, as well as *A. fischeri*, *P. leiognathi*, and *P. mandapamensis*, whereas cold seawater samples plated and incubated at lower temperatures are likely to yield *A. logei*, *P. kishitanii*, *P. phosphoreum*, and *S. hanedai*. It should be noted that some strains of *A. logei* and *S. hanedai* grow well but do not produce light at room temperature; attempts to isolate these and other psychrotrophic bacteria should be carried out at 15 °C. Also, some bacteria, such as *A. salmonicida*, may not produce visible levels of light unless aldehyde is added to the medium (Fidopiastis et al. 1999); a simple screening approach for finding these bacteria is, once the observer is dark adapted, to add a drop of decyl aldehyde to the underside of the lid of the plates used for plating environmental samples and look for previously dark colonies that then become luminous.

Enrichment from fish or squid can be made using fresh samples and sterile seawater or frozen samples with natural, unsterilized seawater (e.g., Ast and Dunlap 2005). The tissue, preferably with the skin on, is placed in a tray, skin up, covered halfway with seawater, incubated, and observed daily in the dark for luminous spots (see *Fig. 13.5*), which arise in one to a few days. These spots, colonies of luminous bacteria, can then be picked and streaked for isolation on the medium described above containing 4 % agar. From fish and squid, a variety of different species of luminous bacteria can be isolated, especially when different incubation temperatures, such as 4 °C, 15 °C, and 22 °C, are used.

Various crustaceans (e.g., gammarid and caprellid amphipods) are suitable sources for luminous bacteria, as they can

become infected with luminous (and nonluminous) bacteria and develop a strong luminescence before and for several hours after dying. In a dark room, after dark adapting for 12–15 min, one can pick out the infected, luminous crustaceans from collected seaweed. In a lighted room, the exoskeleton of the animal is punctured to obtain the hemolymph, which is streaked onto LSW-70 agar plates. The plates can be incubated at ambient or cool temperatures and are observed after 12–24 h for luminous colonies, which are then picked and streaked to obtain pure cultures.

Cultivation

The easily prepared complete medium, LSW-70 (Dunlap et al. 2004), detailed above, is suitable for the growth and luminescence of all culturable luminous bacteria. Most complete marine media, whether prepared with artificial or natural seawater to supply appropriate levels of Na^+, Ca^{2+}, and Mg^{2+}, support the growth of luminous bacteria from marine habitats. Previously, a commonly used complete medium was SWC, prepared with natural seawater diluted to 70 % or 75 %, 5 g per liter of tryptone or peptone, 3 g per liter of yeast extract and 3 ml per liter of glycerol, and with 15 g per liter of agar for solid medium. Traditionally, SWC was buffered with 50 mM Tris or HEPES, or 1 g per liter of solid calcium carbonate was incorporated into the agar medium to control acid production (Nealson 1978). Acid production during growth in SWC, which can lead to death of the cells, results, however, from the presence of glycerol, and elimination of this component avoids the problem (Hill 1928; Johnson and Shunk 1936; Dunlap et al. 1995) with no major effect on growth or luminescence. Nealson (1978) listed and compared various formulations for complete and minimal media. Artificial seawater can be prepared according to the formulation of MacLeod, as described by Nealson (1978), or for routine culture work, a commercial aquarium marine salt mix can be used. Procedures for preparing minimal media have been described by Nealson (1978). Growing marine luminous bacteria in liquid medium may require a low agitation rate, since strong aeration can cause some species to clump at low population density.

Storage

Luminous bacteria have been revived from sealed glass ampoules after more than 80 years and 28 years of storage (Figge et al. 2011; Haneda 1981). Storage of luminous bacteria on agar slants or in agar stabs is suitable for only short periods, that is, days; longer-term storage on media is not recommended, as dim and dark variants easily arise with most species, and survival can be poor. Similarly, survival under refrigeration is poor for some species. Lyophylization or storage in liquid nitrogen may be an option if appropriate equipment is available (Baumann et al. 1984). Perhaps most convenient and effective for retaining strains in their original state is storage at ultralow temperature, for example, −75 °C to −80 °C, in a cryoprotective medium. An effective cryoprotective medium for the storage of luminous bacteria is filter-sterilized double strength deep freeze medium (2X DFM), prepared with 1 % w/v yeast extract, 10 % dimethyl sulfoxide (DMSO), 10 % glycerol, and 02 M K_2HPO_4/NaH_2PO_4 (pH 70). 2X DFM, originally developed by R. Rodriquez for storing yeast, was recommended to us several years ago by E. F. DeLong. Storage using 2XDFM works well for all species of luminous bacteria (P. Dunlap, pers. obs.). For storage of a strain, a dense culture is prepared by growing the strain in a complete liquid medium (e.g., LSW-70 broth) with aeration overnight or longer, to attain a dense population, adding 0.5 ml each of the culture and 2X DFM to cryovials, briefly vortexing to mix, allowing the mixture to stand for 5 min, and then placing the vial into the ultralow temperature freezer. Commercial cell freezing containers containing isopropanol, which allow a slow rate of cooling, work particularly well. Quick freezing in an ethanol bath kept in the ultralow temperature freezer or chilled with dry ice works well, but the ethanol can cause the labeling on tubes to smear. Cultures of luminous bacteria stored in this manner retain viability apparently indefinitely when the vials are kept at constant ultralow temperature.

Identification

The taxonomy of the marine luminous bacteria and their relationships to other marine enterobacteria were established during the 1970s and 1980s through the use of an array of diagnostic physiological, biochemical, and molecular traits (Reichelt and Baumman 1973; Reichelt et al. 1976; Baumann and Baumann 1981; Baumann and Baumann 1981). Very substantial progress was made through this work in clarifying the genus and species diversity of luminous bacteria, and that work established a foundation for understanding the ecological distributions and evolutionary relationships of these bacteria. At a practical level, the use at the time of as few as 10–25 phenotypic traits allowed the identification of many of the commonly encountered species of marine luminous bacteria (Nealson 1978; Baumann and Baumann 1981; Hastings and Nealson 1981).

More recently, however, many of the entities thought to be single species based on these phenetic traits have been found to represent multiple, evolutionarily distinct lineages, that is, separate species and genera, when examined by molecular phylogenetic criteria (e.g., Ast and Dunlap 2005; Ast et al. 2009). Consequently, current methods for the rapid and accurate identification of luminous bacteria and for descriptions of new species increasingly are based on phylogenetic analysis of gene sequences, which is now inexpensive, rapid, and highly accurate. For rapid identifications, sequence analysis of just the *luxA* or *luxB* genes often is adequate for good provisional identifications and can be supplemented or replaced by analysis of the sequence of *gyrB*. A multilocus approach using housekeeping genes such as the 16S rRNA gene, *gyrB*, *pyrH*, *recA*, *rpoA*, and *glnA* has proven very effective for robustly separating closely related luminous bacteria and for revealing the rare instances in which a strain apparently has acquired *lux* genes horizontally.

Information on primers and amplification procedures for these and other genes can be found in Ast et al. (2009), Thompson et al. (2005), Urbanczyk et al. (2008), Fischer Le Saux et al. (1999), and Peat et al. (2010). Together with housekeeping genes, sequence analysis of the *lux* genes has proven particularly valuable for new species descriptions and rapid identification of newly isolated strains (e.g., Ast and Dunlap 2004; Haygood 1990; Ast and Dunlap 2005; Thompson et al. 2005; Ast et al. 2007b; Urbanczyk et al. 2007; Ast et al. 2009).

Complete characterization of a new species of luminous bacteria, however, should include more than just a multigene phylogenetic analysis. Diagnostic biochemical and morphological traits, DNA hybridization analysis, determination of the mol% G + C ratio, fatty acid profile analysis, and comparative genomic analysis such as amplified fragment length polymorphism (AFLP) or repetitive extragenic polymorphic PCR (rep-PCR), in the context of the bacterium's ecology (e.g., Ast et al. 2007a), provide a more complete description suitable for new species. Furthermore, the examination of multiple independent isolates of the new entity and the inclusion in the analysis of the type strains of all closely related and relevant species are critically important for accurate and definitive work.

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