A Luminous Bacterium That Emits Yellow Light

Abstract. *A strain of* Photobacterium fischeri *that emits yellow light has been isolated from seawater. The bimodal spectrum, which is unique among the luminous bacteria, consists of a major band with a maximum at 545 nanometers and a minor band with a maximum at 500 nanometers. The former represents a heretofore unreported range of emission for luminous bacteria, while the latter coincides with the emission spectrum of typical blue-green–emitting strains of* P. fischeri. *The relative contributions of these two bands to the total in vivo luminescence changes as a function of ambient temperature. When luciferase is extracted and luminescence observed in vitro, the emission is entirely blue-green, identical with that of luciferase isolated from other strains of* P. fischeri.

Light is sufficiently unusual as a biological product to have attracted attention and study from many observers over the past century (*1*). The actual number of different isolates of luminous bacteria observed must number in the hundreds of thousands, although those actually subcultured may be fewer by an order of magnitude. Until now, all luminous bacteria have been reported to emit a blue-green light with a peak emission ranging from 472 to 505 nm depending on the strain (*2*). It was therefore surprising both to discover among some new isolates a yellow-emitting luminous bacterium, and to find that it is taxonomically classed as a species previously known to emit blue-green light.

The yellow-emitting bacterial strain was discovered in the course of a systematic study of the taxonomy of luminous isolates from seawater near La Jolla, California. At each sampling, the seawater was plated, the number of luminous colonies on each plate was determined, and the isolates were picked and restreaked to obtain pure, single colonies. The originally isolated colonies are small and dim and are only marginally (if at all) visible by cone (color) vision; however, the streaks are broad and bright, and the yellow and blue-green stains are truly distinct.

On the basis of the classification system of Reichelt and Baumann (*3*), the newly isolated yellow-light–emitting organism (Y-1) belongs to the species *Photobacterium fischeri*. In addition, its luciferase is characterized kinetically as "fast" (*4*), which distinguishes the genus *Photobacterium* from the genus *Beneckea* (*5*). Also, Y-1 produces and responds to autoinducer of the *P. fischeri* type while showing no cross-reaction with inducer of other luminous species. Autoinducer is species-specific (*6*).

The in vivo bioluminescence emission spectra of Y-1 and a typical blue-green–emitting *P. fischeri*, MJ-1, are shown in Fig. 1A, along with the fluorescence emission spectrum of flavin mononucleotide (FMN). In contrast to the typical spectral emission of MJ-1 (a single major blue-green emission peaking at 500 nm), that of Y-1 has a major peak at 545 nm and a shoulder at 500 nm. The in vivo emission spectrum is the same for cells growing either on an agar slab or in a liquid medium. Unlike that reported for some strains (*7*), the spectrum is not affected by cell density but is identical throughout a growth cycle (10^6 to 10^9 cells per milliliter of culture medium). In contrast, in an in vitro assay of Y-1 luciferase, the 545-nm component of luminescence is completely absent—the spectrum exhibits only a single blue-green peak and is the same as that of MJ-1 (Fig. 1B).

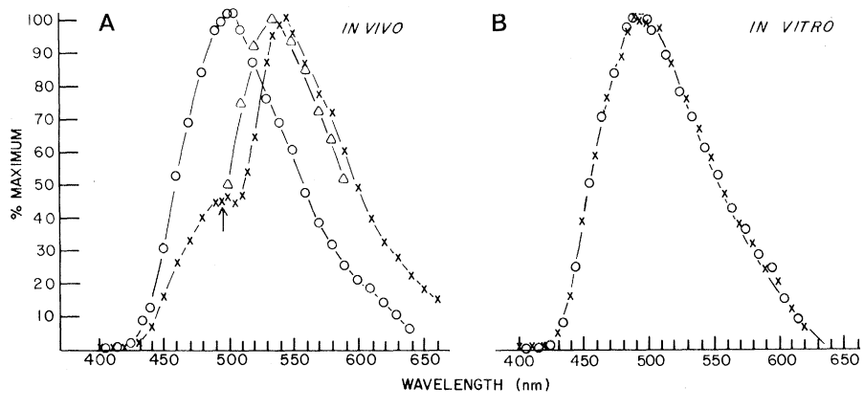

Fig. 1. (A) Emission spectra of bioluminescing cells of MJ-1 (○) and Y-1 (×). Arrow indicates minor peak in the Y-1 spectrum. Cells were prepared for measurement in vivo by growing them on solid medium. A plug of agar was removed and placed in a cuvette facing the entrance split of the emission monochromator of a spectrophotofluorometer (Perkin-Elmer MF-4). The fluorescence emission spectrum of FMN (△) is included for comparison. Spectra presented have been corrected through the use of the known fluorescences of standards [flavin mononucleotide, nicotinamide adenine dinucleotide (NADH), and 8-anilino-1-naphthalene sulfonate]. (B) Emission spectra of cell-free extracts of luciferase from MJ-1 (○) and Y-1 (×), using the coupled assay described by Duane and Hastings (*22*), in 0.01*M* phosphate buffer, *p*H 7, at 22°C. Tetradecanal was routinely used as the aldehyde, because it yielded the most light of the several aldehydes tested. Final concentrations of reagents in the reaction mixture of 1.0 ml were as follows: FMN, $4 \times 10^{-6}M$; tetradecanal, $4 \times 10^{-5}M$; NADH, $7 \times 10^{-6}M$. With this assay, luminescence was constant for at least 5 minutes, which was adequate to scan the spectrum several times. For some experiments, the same assay was performed except that FMN was omitted from the reaction mixture, in which case the flavin in the cell extract was of sufficient concentration to mediate in the reaction with easily measurable bioluminescence (Table 1).

Bioluminescence in vitro is catalyzed by the enzyme bacterial luciferase, an $\alpha\beta$ heterodimeric protein of molecular weight 79,000 with no prosthetic group or metal (*8*). The reaction involves a mixed function oxidation of reduced flavin mononucleotide ($FMNH_2$) and long-chain aldehyde (RCHO) (*9*); the products of this reaction are oxidized flavin (FMN), the corresponding long-chain acid (RCOOH) (*10*), and visible light ($h\nu$).

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH + h\nu$$

We have sought to determine whether a chemical alteration of one or more components of this system gives rise to the yellow emission in vivo or whether some purely physical factor or factors are responsible. The observation that the in vitro spectrum of Y-1 luciferase is identical to that of MJ-1 indicates that the Y-1 enzyme is not significantly altered in its ability to catalyze a typical blue-green light emission. A second possibility is that Y-1 produces an altered flavin (*11*). A concentrated cell extract was obtained from cells grown in a minimal medium (no exogenous flavins). This was assayed both with added FMN and with no added flavin (Table 1). In both cases, only a blue-green emission was observed. Thus either an altered flavin is not responsible for the production of yellow light, or else the flavin for bioluminescence is a minor, luciferase-associated component that is removed during enzyme preparation and replaced by cellular FMN.

To test the possibility that cellular aldehyde might be involved, dark mutants of both MJ-1 and Y-1 were isolated after treatment with *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (*12*). Among those isolated were mutants that are nonluminous

unless the cells are exposed to long-chain aldehydes (*13*). When such mutants are exposed to decanal vapor, they respond with luminescence of a color characteristic of that of the parent: MJ-1 gives blue light and Y-1 gives yellow light (Table 1). Thus, the cellular aldehyde is not essential to the production of an altered emission.

Since there was no evidence for the presence in Y-1 of a modified component of the in vitro reaction sequence that could be responsible for the shift to yellow emission, several other factors were considered. We attempted to detect the presence of a screening pigment in Y-1 by passing the light from a cell suspension of MJ-1 through a culture of Y-1 aldehyde (dark) mutants. The spectrum of the transmitted light was identical to that of the incident MJ-1 emission; that is, there was no selective absorption by the Y-1 cells.

Although we found no indication of a screening pigment, there was evidence for a direct energy transfer from the excited product to another molecular species that serves as the emitter. Such a mechanism, which would lead to an emission of longer wave length, has been demonstrated in coelenterate bioluminescent systems (*14*). The facts that the in vitro reaction is blue and that this emission band is also a component in vivo suggest that an excited state, which can lead to blue emission, is the primary product. The possibility that energy transfer to a second emitter could account for the yellow emission is a hypothesis supported by additional observations on the relative intensities of the two bands. Under certain conditions, such as a shift in temperature, the relative intensities of the two bands shift reciprocally; when the intensity of the yellow increases, that of the blue decreases and vice versa. At 20°C or below, the ratio of light intensities at 545 and 490 nm is approximately 2.1 : 1. A marked change occurs between 23° and 25°C with the blue-green component increasing and the yellow decreasing with increasing temperature. At 25°C and above, the ratio drops to 1 : 1 or less, and the light appears noticeably more blue to the eye. If the temperature is again lowered to 20°C, the emission spectrum returns to the yellow pattern of Fig. 1 within minutes. This effect is readily explained if the efficiency of energy transfer can be altered by temperature, but it is not consistent with a model dependent on pigment screening.

The energy of electronically excited states can be transferred both to identical (homotransfer) and to alternate (heterotransfer) molecular species in chemical systems as described by Förster (*15*) and Stryer (*16*). A major consideration in such reactions is the spatial relationship of the two molecules, because the transfer efficiency falls off with the sixth power of the distance in Förster-type transfer. The acceptor species can be postulated to be a membrane component adjacent to the luciferase on the assumption that in vivo the latter is associated with certain membrane proteins and components of the electron transport system (*17*). Upon cell lysis, luciferase is released into the soluble fraction and diluted away from the acceptor molecule. Thus, efficient energy transfer can no longer occur. To search for a possible candidate for the role as the acceptor molecule, a cell suspension of the dark Y-1 aldehyde mutant was used. An excitation spectrum for emission at 570 nm was made; two major peaks were demonstrated, one at 380 nm, the other at 475 nm. Exciting the cell suspension at 475 nm yielded a bimodal emission spectrum similar to that in Fig. 1. Thus, Y-1 does contain a compound that absorbs in the blue-green and emits in the yellow.

Table 1. Emission peaks of bioluminescence spectra (in nanometers) of two strains of *Photobacterium fischeri*; N.O. indicates that no peak was observed.

Bacterial strain	Medium	Assay condition	Y-1		MJ-1	
			Major	Minor	Major	Minor
Wild type	Complete	In vivo	545	500	500	N.O.
		In vitro	495	N.O.	495	N.O.
		In vitro (no FMN)	495	N.O.	495	N.O.
Wild type	Minimal	In vivo	545	500	500	N.O.
		In vitro	495	N.O.	495	N.O.
		In vitro (no FMN)	495	N.O.	495	N.O.
Aldehyde mutants	Complete	In vivo (with aldehyde)	545	500	500	N.O.

Energy transfer in bacterial luminescence is a novel hypothesis and carries with it the notion that FMN is not the in vivo emitter. If energy transfer occurs and is subject to spatial perturbation, such a mechanism could account for the fact that in vivo emission spectra of bioluminescent bacteria have been reported to vary from 472 to 505 nm although the enzyme emission spectra in vitro are generally similar (*2*). The strain Y-1 may merely represent an extreme case of a mechanism normally occurring in bacterial bioluminescence. If so, determining the molecular components responsible for the yellow emission should further elucidate the in vivo light-emitting system in the luminous bacteria.

Photobacterium fischeri has been identified as a symbiont in at least two marine fishes, *Monocentris japonica* and *Cleidopus gloriamaris* (*18*). Both these and other symbioses of luminous bacteria and marine fishes have been shown to be species-specific (*18, 19*). The fact that the species *P. fischeri* includes members that can emit either blue-green or yellow light suggests that the mechanism whereby specificity of symbiosis is maintained may be more sophisticated than the taxonomic characters used to define species would indicate. If the major niche of *P. fischeri* is in fact that of a symbiont, then the occurrence of Y-1 may indicate the existence of host organisms (for example, fishes or squids) that emit or utilize yellow light. So far, such organisms have not been found in the marine environment. The fact that all previously isolated bacteria emit a blue-green light (which is well transmitted through seawater and universally seen by marine photoreceptors) has suggested that the light itself is the useful item (*1*). There are, of course, other possible explanations for the presence of bioluminescence if the emission of a particular color is not strongly selected for. The light may simply have no selective advantage (*20*), or the light-emitting system may function in some biochemical sense (*21*) in which the emission of the light per se (and, more specifically, the light of a particular color) would not be selected for. The strain Y-1, or future isolates like it, may thus be of great value not only in elucidating the mechanism of in vivo bacterial light emission, but in understanding the ecology of the luminous bacteria and of bioluminescence in general.

EDWARD G. RUBY
KENNETH H. NEALSON
Scripps Institution of Oceanography, La Jolla, California 92093

References and Notes

1. E. N. Harvey, *Bioluminescence* (Academic Press, New York, 1952).
2. H. H. Selinger and R. A. Morton, in *Photophysiology*, A. C. Giese, Ed. (Academic Press, New York, 1968), vol. 4, p. 253.
3. J. L. Reichelt and P. Baumann, *Arch. Mikrobiol.* **94**, 283 (1973); *J. Syst. Bacteriol.* **25**, 208 (1975).
4. J. W. Hastings and G. W. Mitchell, *Biol. Bull.* **141**, 261 (1971).
5. We have examined more than 2500 natural iso-

lates and culture collection strains of luminous bacteria for taxonomic identity and luciferase kinetics. In all cases, members of the genus *Photobacterium* have the "fast" mode of decay kinetics, whereas luminous *Beneckea* isolates have the "slow" mode.
6. J. Magner, A. Eberhard, K. Nealson, *Biol. Bull.* **143**, 469 (1972).
7. A Spruit-van der Burg, *Biochim. Biophys. Acta* **5**, 175 (1950).
8. J. Friedland and J. W. Hastings, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 2336 (1967).
9. K. H. Nealson and J. W. Hastings, *J. Biol. Chem.* **247**, 888 (1972).
10. O. Shimomura, F. H. Johnson, Y. Kohama, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2086 (1972); D. K. Dunn, G. A. Michaliszyn, I. G. Bogacki, E. A. Meighen, *Biochemistry* **12**, 4911 (1973); F. McCapra and D. W. Hysert, *Biochem. Biophys. Res. Commun.* **52**, 298 (1973); A. Vigny and A. Michelson, *Biochimie* **56**, 171 (1974).
11. G. Mitchell and J. W. Hastings, *J. Biol. Chem.* **244**, 2572 (1969).
12. T. W. Cline and J. W. Hastings, *Biochemistry* **11**, 3359 (1972).
13. P. Rogers and W. D. McElroy, *Proc. Natl. Acad. Sci. U.S.A.* **41**, 67 (1955); K. Nealson and A. Markovitz, *J. Bacteriol.* **104**, 300 (1970); T. W. Cline and J. W. Hastings, *J. Biol. Chem.* **249**, 4668 (1974).
14. J. W. Hastings and J. Morin, *Biochem. Biophys. Res. Commun.* **37**, 493 (1969); J. Morin, in *Coelenterate Biology*, L. Muscatine, H. M. Lenhoff, Eds. (Academic Press, New York, 1974), p. 397; ——— and J. W. Hastings, *J. Cell. Physiol.* **77**, 305 (1971); M. J. Cormier, J. Lee, J. E. Wampler, *Anna Rev. Biochem.* **44**, 255 (1975).
15. T. Förster, *Naturwissenschaften* **33**, 166 (1946); *Discuss. Faraday Soc.* **27**, 7 (1959).
16. L. Stryer, *Science* **162**, 526 (1968).
17. The enzyme bacterial luciferase has in the past been considered to be a soluble enzyme [B. Strehler, *J. Am. Chem. Soc.* **75**, 1246 (1953)] because it is found in the supernatant of lysed cells. Recently Z. Ne'eman, S. Ulitzur, D. Branton, and J. W. Hastings, (*J. Bacteriol.*, in press) have shown that several membrane-bound proteins are induced simultaneously with the induction of luciferase. Thus, it may be that, in intact cells, luciferase functions in association with these membrane proteins.
18. E. G. Ruby and K. Nealson, *Biol. Bull.* **151**, 574 (1976)
19. J. L. Reichelt, K. Nealson, J. W. Hastings, *Arch. Microbiol.*, in press.
20. H. H. Seliger and W. D. McElroy, *Light: Physical and Biological Action* (Academic Press, New York, 1965).
21. K. Nealson, A. Eberhard, J. W. Hastings, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1073 (1972).
22. W. Duane and J. W. Hastings, *Mol. Cell. Biochem.* **6**, 53 (1975).
23. This research was supported by grant PCM 74-14788 from the National Science Foundation.

23 September 1976; revised 15 November 1976

Chemotaxis of *Rhizobium spp.* to a Glycoprotein Produced by Birdsfoot Trefoil Roots

Abstract. Rhizobium spp. *show chemotaxis to plant root exudates. A glycoprotein has been isolated from the root exudates of birdsfoot trefoil,* Lotus corniculatus, *which, at micromolar concentrations, attracts six strains of rhizobia. This glycoprotein has been given the trivial name trefoil chemotactin and contains approximately twice as much protein as carbohydrate. Gel filtration of trefoil chemotactin on a Bio-Gel A-1.5m column gave a molecular weight of approximately 60,000. Trefoil chemotactin represents a new class of chemoattractants for bacteria.*

Nitrogen availability is critical for agriculture since available fixed nitrogen often limits field crop productivity. Commercial nitrogen fixation consumes large quantities of fossil fuels and energy (*1*). Biological nitrogen fixation uses the energy of sunlight and atmospheric nitrogen.

The genus *Rhizobium* is characterized by its ability to elicit nodules and fix nitrogen on legume roots. The bacterium-host interaction is specific in that a single strain of *Rhizobium* can infect only certain species, or even certain varieties within a species, of legumes (*2*). In fact, rhizobia are classified by their host range. The basis of this bacterium-host specificity is unknown.

It is known that the numbers of an infective strain of *Rhizobium* are selectively increased in the rhizosphere of a legume (*3*). Before nodulation the surface of the root is covered with a matrix of bacteria (*4*). Munns (*5*) showed that in liquid culture more bacteria accumulated on alfalfa roots in the first few hours after inoculation than could be accounted for by multiplication of the inoculum. Since rhizobia are generally motile (*6*), we set out to determine if chemotaxis of rhizobia to legume roots might play a part in nodulation. Earlier work in our laboratory showed that six strains of *Rhizobium* show differential chemotaxis to root exudates of legumes and nonlegumes (*7*). That is, a single strain of *Rhizobium* is attracted to some plant root exudates and is unaffected by others.

Crude birdsfoot trefoil root exudates attract trefoil *Rhizobium* strain 95C13 (*7*). Root exudates were collected from 10-day-old seedlings grown under sterile conditions in medium that contained no combined nitrogen. Surface-sterilized seeds were suspended above the liquid medium on an aluminum screen covered with cheesecloth. Clear root exudates were collected and filtered. Fractions of the root exudates were tested for fungal and bacterial contamination by plating on enriched media; no colonies were seen (*7*).

Chemotaxis was measured by counting the radioactivity in bacteria labeled by growth on [U-^{14}C]glucose that swam into a 5-μl micropipette dipped in a suspension of labeled bacteria (*7*). The micropipette contained nitrogen-free medium as control or a fraction of trefoil root exudate. Five replicates were used for each sample solution.

The work we now report was directed toward isolation and characterization of the compound in trefoil root exudate responsible for its attraction of trefoil *Rhizobium*. Treatment of trefoil root exudate with pronase or acid completely destroyed its ability to attract trefoil *Rhizobium* (*8*). Boiling the exudate for 10 minutes did not affect the activity. These results indicate that the active principle in the root exudate, which we have called trefoil chemotactin, is heat stable and contains peptide bonds.

The crude trefoil root exudate (1 liter) was concentrated tenfold, and two volumes of cold acetone were added. The precipitate was removed by centrifugation and the supernatant liquid, containing chemotactin, was reduced in volume 30-fold. Higher concentrations of acetone would precipitate chemotactin; however, it was difficult to resolubilize in water or buffer from the pellet. Hence, the acetone-soluble fraction (67 percent acetone) was extracted twice with double the aqueous volume of *n*-butanol, and then in the same manner with chloroform, and finally with ether. The

Table 1. Chemotaxis of the trefoil strain of *Rhizobium* to fractions of a birdsfoot trefoil root exudate. The chemotactic ratio, R, is calculated as the counting rate (disintegrations per minute) of the sample divided by that of the medium (control). The chemotactic ratio divided by the carbon content, R/CC is used as a measure of specific activity.

Test solution	Average disintegrations per 10 minutes	R	Carbon content (μg/ml)	R/CC
Nitrogen-free medium	258			
Concentrated trefoil root exudate*	689	2.67	3442	7.76×10^{-4}
Acetone supernatant	1080	4.19	2112	1.98×10^{-3}
Bio-Gel P-2 void volume	1052	4.08	141	2.89×10^{-2}
Ion exchange neutral	1950	7.56	266	2.84×10^{-2}
Bio-Gel A-1.5m, peak I	3148	12.20	26	4.69×10^{-1}

*The contents of ten 1-liter beakers—a total of approximately 800 ml of root exudate from 500 to 1000 10-day-old seedlings—were combined, filtered, and concentrated. A portion was lyophilized and assayed at each step of the purification (see text).