Luminescence 1999;14:147–151 *ORIGINAL RESEARCH*

Comparison of relative photon flux from single cells of the bioluminescent marine bacteria *Vibrio fischeri* and *Vibrio harveyi* using photon-counting microscopy

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ABSTRACT: Photon counting microscopy was used to quantify relative photon flux from 616 single sessile cells of *Vibrio harveyi* and 180 single sessile cells of *V. fischeri*. *V. fischeri* cells all emitted light at nearly the same level (average 15.93 ± 11.48 photons/min, range 0–58.9 photons/min) when bioluminescence was induced, whereas photon flux of *V. harveyi* single cells was extremely variable (average 10.51 ± 20.60 photons/min, range 0–211 photons/min).

KEYWORDS: photon-counting; microscopy; *Vibrio harveyi*; *Vibrio fischeri*; confocal microscopy; bioluminescence

INTRODUCTION

Vibrio fischeri and *Vibrio harveyi* are symbiotic marine bacteria (1); *V. fischeri* occurs in the light organs of fishes and invertebrates, whereas *V. harveyi* is commensal in digestive tracts and on the surfaces of fishes and hard-bodied invertebrates. Both species are naturally bioluminescent when autoinduction of their *lux* genes occurs. This autoinduction is due in part to the phenomenon of quorum sensing, whereby population density is coupled to transcriptional activation by the action of N-acyl homoserine lactones (NAHLs)—diffusible chemical signals with roles in cell-to-cell communication in many Gram-negative bacteria and which regulate light production by these *Vibrio* species (2). Quorum sensing, autoinduction and *lux* regulation are subjects of intense research because of their general applicability to understanding signalling in bacterial cells (2–4). Additional interest is focused on bioluminescence in construction of bacterial reporters (5, 6).

Most assays of bioluminescence have relied on measurements obtained with luminometers (7) or scintillation counters, and have assumed that photon flux is uniform across all single cells in a population. Some studies have addressed the bioluminescence of single cells (8), but most microscopic studies have either failed to show colocalization of a light source and a single cell (9, 10) or have been qualitative (11). Exceptions are the work of the present group (12) and some studies on genetically engineered bacteria (13, 14).

Studies of *Vibrio* spp. are ordinarily performed in liquid culture, a condition that does not reflect the attached state of the bioluminescent cell (15). In our hands, *V. fischeri* (ATCC strain 7744) batch cultures were visibly more luminescent than were those of *V. harveyi* (ATCC strain 14126). This led to the initial assumption that the strain of *V. fischeri* cells had higher photon flux than did the strain of *V. harveyi*. However, when attached single cells were examined, it became apparent that this assumption was incorrect.

MATERIALS AND METHODS

Bacterial strains, culture conditions and induction of bioluminescence

V. harveyi ATCC strain 14126 and *V. fischeri* ATCC strain 7744 were obtained from the American Type Culture Collection (Rockville, MD). Both species were grown at 23°C in 50 ml shaken batch cultures for 15–18 h in Seawater Complete (5 g tryptone, 3 g yeast extract, 3 ml glycerol, 750 ml seawater, 250 mL distilled water). This growth period yielded maximum light output, as determined visually in a dark room. Spent medium (containing NAHLs) was sterilized by filtration (pore diameter 0.2 μm). Aliquots of the original culture (200 μL) were diluted in 4.0 mL of spent medium to obtain a concentration of approximately 10^6–10^7 cells/ml. This suspension was then injected into a microscopy flowcell (two coverslips separated by a silicone spacer; 250 μL total volume) (16, 17). The inoculated flowcell

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was incubated at room temperature under static conditions for 20–60 min before spent medium was perfused through the flowcell to remove unattached cells and induce the *lux* cassette. Autoinducer was removed by perfusion with dilute (1/10), sterile growth medium. The flow rate of the bulk fluid ranged from 20 to 100 μL/min. This inoculation procedure resulted in monolayer biofilms in which single cells were spatially separated.

Microscopy and photon detection

Transmitted-light and photon-counting images were obtained with a Hamamatsu VIM3 camera and Argus 50 control software (Hamamatsu Photonics, NJ). The camera was mounted on a Zeiss Axioplan or a Leica DMR microscope and cells were observed with a 100 × 1.3 N.A. oil-immersion lens. Photons emitted by bacterial cells travel through the objective lens, strike a photocathode in the image intensifier and are converted to photoelectrons. Photoelectrons then enter a multichannel plate where up to 10^6 amplification occurs. The photoelectron bursts leaving the multichannel plate-strike a phosphor plate, and are converted back to photons, which are focused on a charge-coupled device camera whose control software maps each original photon to 1 pixel in the image. An indexed colour image encodes the number of photons/pixel over time. Software permits quantitative analysis of user-defined areas within the photon-counting image to determine photon flux from a single cell. Transmitted-light images of the same field of view were obtained to correlate light production with bacterial location (18).

The instrument has not been calibrated with a standard light source because of difficulty in obtaining a point-source of light of the size of a bacterium. Although unpublished results from this laboratory suggest that chemiluminescence of alkaline-phosphatase-coated microbeads may provide such a standard, absolute photon fluxes cannot presently be obtained because no correction for system losses (absorbance by medium, coverglass, immersion oil, or incomplete entrance to the multichannel plate) can be made using relevant samples. The efficiency of detection will, however, be the same within these experiments and the numbers are sufficient for comparative purposes.

Photon counting images were accumulated over periods of 5–20 min. Data are expressed in photons/min within each accumulation period. Background photon levels were determined by counting the number of photons in an area in each field of view farthest from any attached cell, and were subtracted from the total number of photons emitted from each cell. Cells with photon counts equal to or below background were assigned an emission value of zero. The apparatus was contained in a light-tight box to reduce background from ambient light.

Viability assay

Cellular viability was determined by staining with Live/Dead® *Bac*Light™ Bacterial Viability Kit for microscopy (Molecular Probes, Eugene, OR) after photon-counting images were obtained. Staining mixture (SYTO 9 and propidium iodide) was prepared in sterile Seawater Complete medium according to the manufacturer's directions. Viable cells (stained with SYTO 9) emit green fluorescence and non-viable cells (stained with propidium iodide) emit red fluorescence. An aliquot (1 mL) of the mixture was injected into the flowcell. Confocal micrographs were obtained before and after addition of ethanol (200 μL, 70%) to the bulk fluid. Images were obtained with a Leica laser confocal microscope (TCS-NT) (Heidelberg, Germany) with 100×, 1.4 NA oil-immersion lens. An argon laser (488 nm) served as the excitation source for both fluorophores.

Statistical analyses

Statistical parameters and tests were analysed and performed using the SAS System for data analysis (SAS Institute Inc., Cary, NC). Significant difference in mean photon flux was determined by a two-sample *t*-test.

RESULTS

Comparison of photon flux between species

Nearly all *V. fischeri* cells emitted photons when autoinducer was perfused through monoculture biofilms.

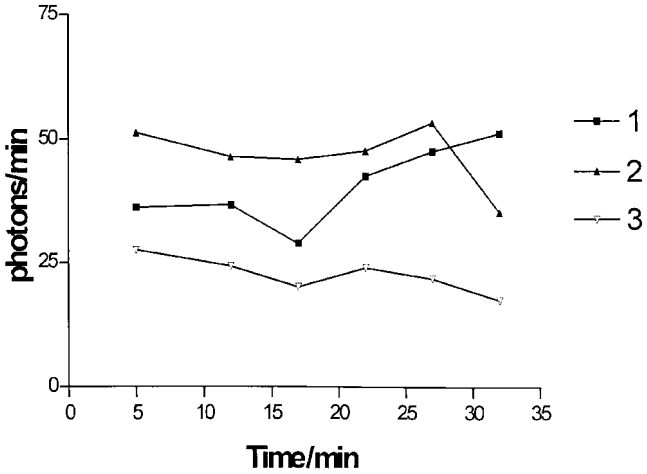

Figure 1. Three single *V. fischeri* cells from one field of view exhibit similar photon flux during a 35-min time-course experiment. Light-producing cells were allowed to attach to the glass surface (coverslip) of a flowcell and cell-free medium containing autoinducer was pumped through. Light production rates of single cells were relatively constant over the 35-minute period

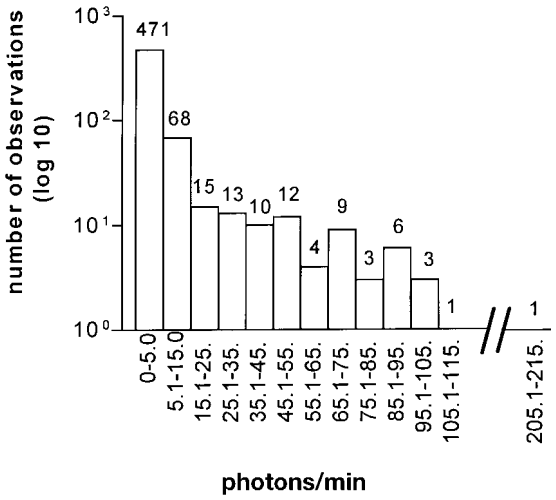

Figure 2. Frequency distribution for *V. harveyi* photon flux. Note the large number of cells with low photon flux (0–5.0 counts/minute/cell) and the wide range of light output. The number of observations for each photon flux range are indicated above bars. Zero cells were observed with flux in the range 115.1–205.0

Furthermore, these cells produced light at nearly the same level: between 10 and 50 photons/min (Fig. 1). *V. fischeri* exhibited a mean photon flux of 15.93 ± 11.48 ($n = 180$) photons/min. In contrast, the majority of attached *V. harveyi* cells did not emit any photons, or did so at levels barely above background (Fig. 2). The mean photon flux of *V. harveyi* cells was 10.51 ± 20.60 ($n = 616$) photons/min. Unequal variances between the two sample populations led to use of an approximate two-sample *t*-test to compare means. The mean photon fluxes between the two species are different at the 1% significance level.

Variability of photon flux between single cells of the same species

Representative transmitted light and photon-counting images for both species are shown in Plate 1. Although some *V. harveyi* cells were attached only at the cell pole, most were orientated horizontally immediately at the coverslip (Plate 1A); photon-flux was determined only for horizontally orientated cells. Only a small minority of *V. harveyi* cells emitted a high number of photons/min (Plate 1B, yellow/red regions). *V. fischeri* cells, in contrast, produced light rather uniformly (*cf.* Plate 1B and 1D). Of 616 *V. harveyi* cells, 27 (4%) had photon counts higher than the highest count determined for any *V. fischeri* cell. Fifteen of these *V. harveyi* outliers were greater than three interquartile ranges from the sample mean (Fig. 3). Thus, single-cell light-production by *V. harveyi* varied over a wide range, but many cells produced no light. In contrast, light production by *V. fischeri* cells was consistent within a relatively narrow range and most cells produced light.

Cellular viability (Plate 2)

A viability assay was performed to ensure that non-luminescing *V. harveyi* cells were viable. Photon-counting micrographs were obtained in which a *V. harveyi* microcolony (15–40 cell aggregate) and single cells were present (Plate 2D). Live/dead staining and

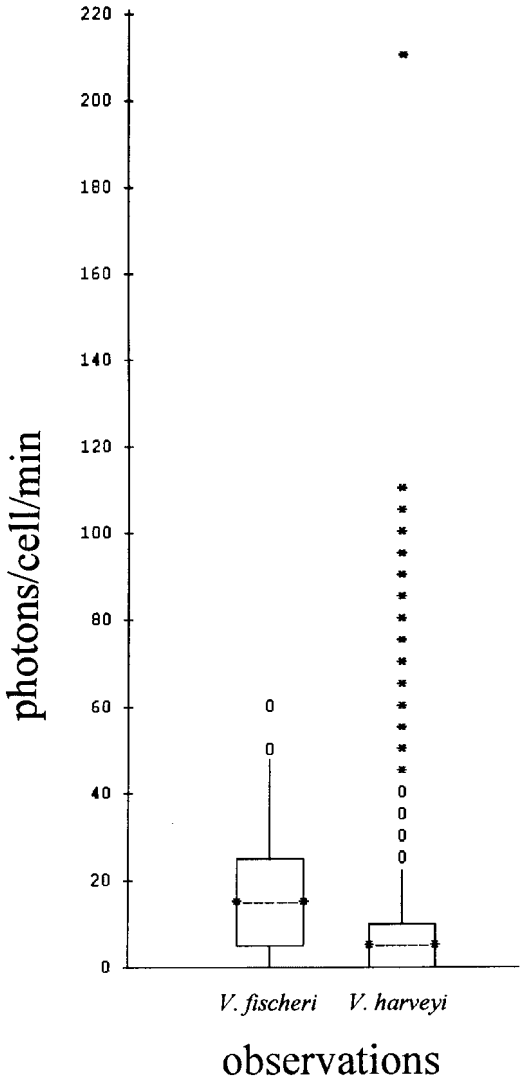

Figure 3. Statistical treatment of photon flux data from 617 *V. harveyi* cells and 180 *V. fischeri* cells. Side-by-side box plots of photon flux from *Vibrio* cells. The lower and upper sides of the boxes indicate the 25th and 75th percentiles. The total box length is one interquartile range. Dashed lines within boxes indicate medians and have asterisks at each end for emphasis. Central vertical lines extend 1.5 interquartile ranges from box edges to the first outlier. Outliers are indicated by a zero if they lie within 1.5 and 3 interquartile ranges of the box. Values beyond that range are indicated by asterisks. Data for *V. harveyi* is that plotted in Fig. 2; comparison will help with interpretation of Fig. 4. *V. harveyi* cells show greater variation (wider range) of light output than do *V. fischeri* cells; however, *V. fischeri* cells have a higher average light output per cell. Especially important is that many *V. harveyi* cells emitted no light (box extends to zero; *cf*. Plate 1D)

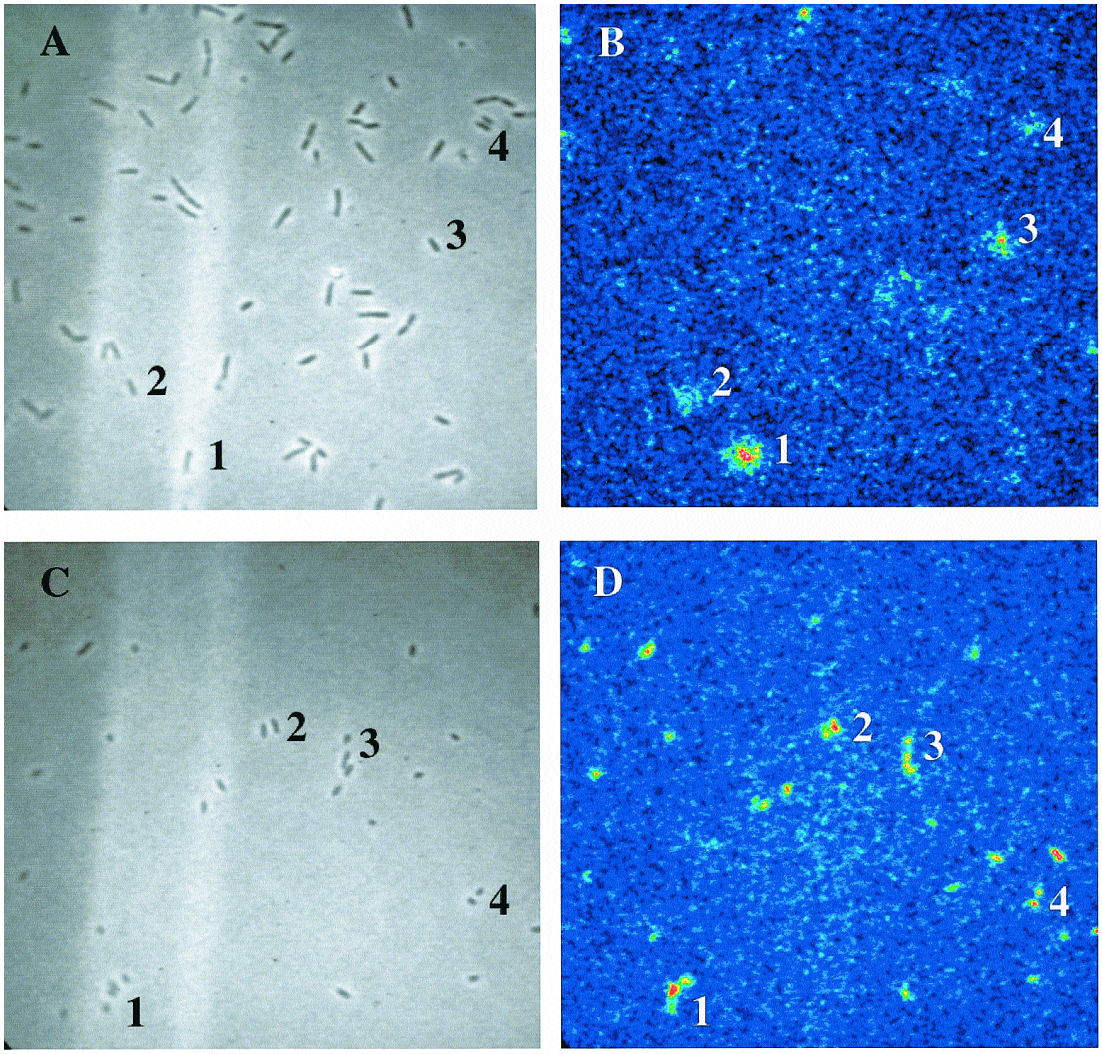

Plate 1. Transmitted-light (A,B) and photon-counting (slice mode) images (C, D) of *Vibrio* species. Transmitted-light images were acquired immediately prior to the photon-counting images. Numbers show regions of correlation (single cells) in each field of view. (A) Transmitted-light image of single *V. harveyi* cells. These cells are attached to the glass substratum and spent medium containing NAHLs is being perfused. Most cells are oriented horizontally; photon-counting was performed only on horizontally oriented cells. (B) Photon-counting image (slice mode) of same field as shown in (A). Colour shifts from blue to red as more photons are detected. Note variation in photon flux between individual cells (2 and 4 *vs*. 1 and 3). (C) Transmitted-light image of single *V. fischeri* cells. These cells are attached and spent medium containing NAHLs is being perfused. All cells are oriented horizontally at the coverslip. (D) Photon-counting image of the same field as shown in (C). Many cells are producing light and almost all of those cells have red regions (*cf*. B)

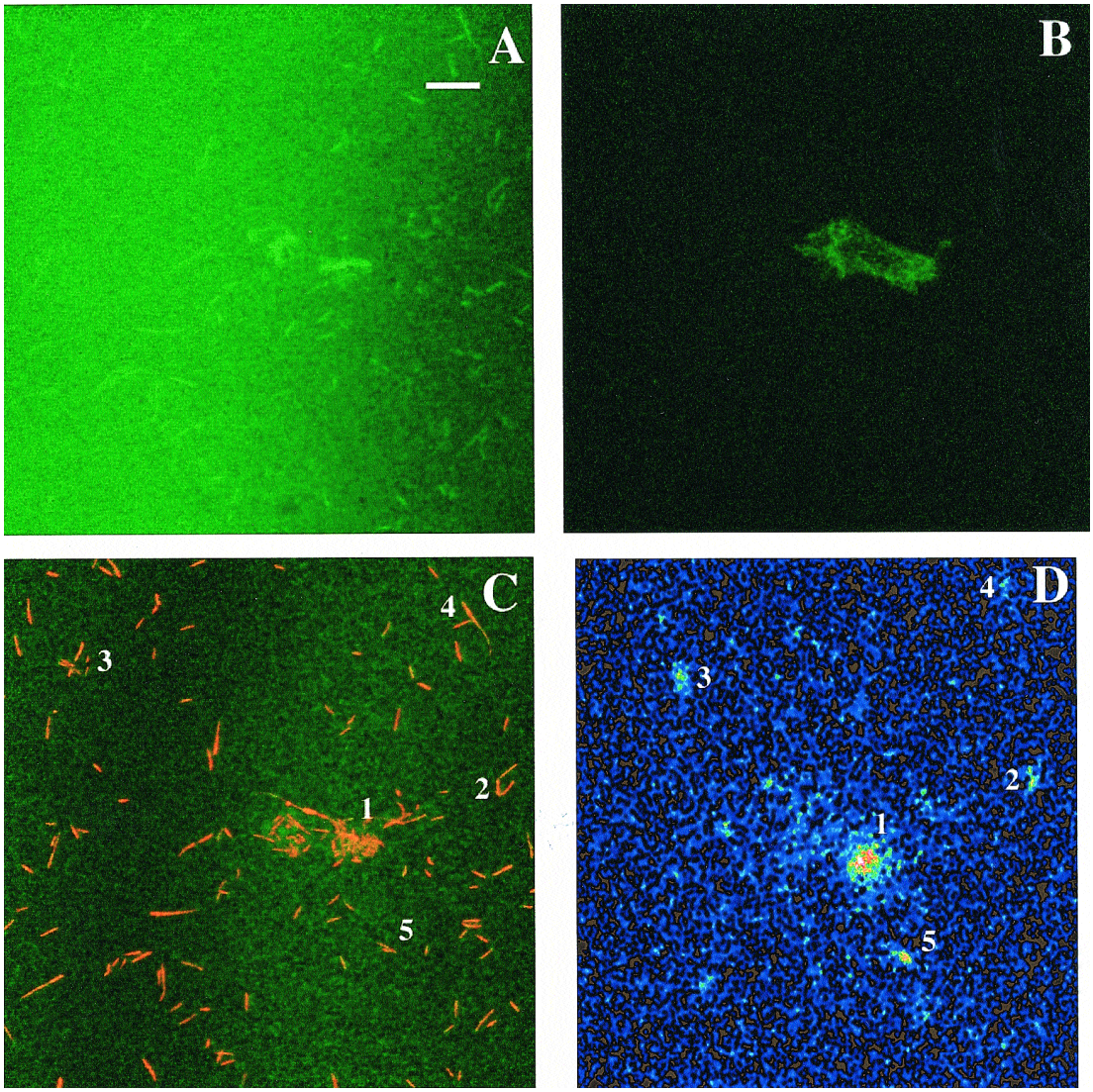

Plate 2. Confocal microscopy of stained cells showing viability of all *V. harveyi* cells, including those not producing light. All confocal micrographs are overlays of green and red channels and show Syto-stained cells (green; viable) and propidium iodide-stained cells (red; non-viable) in each field (scale bar = 10 μm). (A) Extended focus image through 0.8 μm directly at the coverslip. Individual *V. harveyi* cells are visible although difficult to discern over the background staining of the coverslip. All cells are viable (green). (B) Projection image through 8 μm directly above the coverslip. Microcolony of *V. harveyi* cells is easily discernible. The coverslip background has been eliminated and single cells at the substratum do not appear in this image because the depth-of-field does not include the coverslip. Single calls within the microcolony are not easily discernible. No propidium iodide-staining is visible. (C) After exposure to ethanol, all cells are stained by propidium iodide. Projection image through 9 microns including the coverslip region. Numbered areas are regions that correspond with areas of light emission in (D). (D) Photon-counting image of field prior to staining. Field-of-view is marginally smaller than in confocal images

confocal imaging revealed that all cells were viable: Plate 2A shows only those cells immediately at the coverslip, whereas Plate 2B shows all cells within the microcolony). Addition of ethanol to the bulk fluid resulted in killing, as demonstrated by propidium iodide penetration in all cells (Plate 2C). Comparison of Plate 2D with the other panels shows that cells that did not produce light were viable, and that all cells responded uniformly to ethanol killing.

DISCUSSION

When coupled with a high-magnification, high NA lens, the photon-counting camera has clear advantages for study of bacterial bioluminescence. Co-localization of cells with light sources provides unequivocal quantitative detection of photon flux from single cells. We have demonstrated that many cells do not produce light and this must be taken into account in studies that fail to demonstrate co-localization. It would be useful to know if other species of luminescent bacteria have large variations in luminescence from cell to cell.

Differences in photon flux observed in these two species have implications for the ecological niche of each bacterium. One *V. fischeri* mutualism is that formed with *Euprymna scolopes*, the bobtail squid (19). The bacterium colonizes the light organs of juveniles and may form pure cultures within that organ. Bioluminescence in the particular *V. fischeri* strain from *E. scolopes* cannot be detected by the naked eye in pure culture, but in the squid the light production is easily discernible. The explanation for this dicrepancy was shown to be underproduction of autoinducer in culture (20); cultured bacteria responded to exogenously introduced autoinducer and had a functional *luxI* gene. Further, it has been shown that autoinducer concentrations within the light organ of the host are sufficiently large to induce bioluminescence and that autoinducer diffuses freely across the membranes of the organ and its associated bacteria (21). Therefore, it appears that low production of autoinducer by those bacteria in culture does not play a role in the bacterium's response in nature and we suggest this may be true for all mutualistic strains. Light production within the organ may be controlled by other factors (host-related) and therefore we suggest that a very uniform response within the bacterial population is advantageous to the host. Thus, the more uniform photon flux we observed between single cells of the mutualistic bacterium might be expected because the regulatory response of these cells is less broad. The functions of *V. harveyi* bioluminescence are less clear. This species establishes no specific associations but does colonize the intestinal tracts, skin and gills of numerous marine organisms (1), as does *V. fischeri* also. Light produced by bacteria within excretions or on the host itself may attract predators and scavengers (22). It has been suggested that this may lead to reingestion into the new host's intestinal tract where nutrients are available and conditions are suitable for proliferation (22), although direct experimental evidence for the attraction/ingestion function of bioluminescence is lacking (23).

It is possible that autoinducer synthesis induced by quorum sensing may serve other purposes. Perhaps this is a mechanism for signalling location, or to indicate conditions at a location, to other bacterial cells. A competitive advantage may be gained by a species if induction of attachment or detachment from the host ultimately leads to relocation a site to where conditions are more suitable for growth. However, this does not explain the purpose or relevance of light production. Autoinducer induces bioluminescence and its own further production. Within a luminescent microcolony, autoinducer could be present at higher concentrations than in the bulk fluid due to diffusional constraints within the cell aggregate. Microcolony formation is dependent upon conditions suitable to growth. It is reasonable to hypothesize that other cells might respond to a gradient of autoinducer resulting in relocation.

If photon emission by single cells in batch culture mimics that observed here in biofilms, then our original observation of higher visible luminescence in *V. fischeri* batch cultures vs. those of *V. harveyi* is explained. It appears that single *V. fischeri* cells do not emit more photons than do those of *V. harveyi*. Rather, nearly all *V. fischeri* cells emit photons whereas most *V. harveyi* cells do not.

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