Observation of super-Poisson statistics of bacterial (*Photobacterium phosphoreum*) bioluminescence during the early stage of cell proliferation

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We studied the process of the analysis of photon statistics for extraction of physiological information from chemiluminescence accompanying biological phenomena implicated in metabolic processes. A super-Poisson behavior in the early stages of cell proliferation of marine luminescent bacteria was observed by characterization of Fano factor of detected photoelectron time series. It is suspected that there is clustering of photon emission at the elementary luminescence process generated by the activation of luminescent enzymes. [S1063-651X(98)07902-1]

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I. INTRODUCTION

Characterizing the quantum stochastic properties of an optical radiation field provides information concerning the elementary process that comprises the excitation and photon emission processes. From the semiclassical aspect, an optical radiation field is characterized as a chaotic field accompanying a Gaussian intensity fluctuation and a coherent field with a deterministic intensity. However, if radiators do not behave in a statistically independent manner, an intensity fluctuation is expected to show distinct properties compared to the chaotic field. Likewise the quantum theory of radiation fields describes nonclassical light, such as quadrature phase squeezed states or photon-number squeezed states [1], with characteristics of antibunting of photoelectron events or sub-Poisson statistics of the number of photoelectrons [2].

On the other hand, the light emission system underlying living organisms is known as bioluminescence and ultraweak photon emission, referred to as biophoton emission [3,4]. These phenomena are incorporated into the metabolic processes of life, and their properties reflect intensity fluctuations originating from the interplay among elementary biochemical reactions under physiological conditions. A biological system is understood as a highly integrated organization from a subcellular molecular level up to a macroscopic level, illustrated as the hierarchy structure. It was believed that complex regulation circuitry and interactions over the hierarchy emerge as macroscopic spatial, temporal, or functional structures, expressed as self-organizing or self-regulating. Hence, photon emission properties that are derived from biochemical excitation processes will carry information regarding feedback or Markovian reaction processes that consist of elementary reactions. For that purpose, the analysis of photon statistics or photon correlations are effective and these techniques have been utilized to characterize the light scattering process or photon emission from chemiluminescence [5]. We studied the methodology for the analysis of photon statistics of ultraweak light spontaneously emitted from chemically excited states within a biological system, based on the measurement of a time series of photoelectron pulses.

The marine luminous bacteria is one of the well-known bioluminescent organisms. Its emission mechanism is based on an enzymatic (luciferase) oxidation process with high a quantum yield (total $\sim 10\%$) of light emission [6,7]. For example, in the case of *Vibrio fischeri*, a self-generated autoinducer [8] activates the transcription of the luminescence (*lux*) genes, which encode proteins for autoinducer synthesis and light emission; thus light intensity depends on the population density of cells [7]. It is also known that *Photobacterium phosphoreum* strain IFO13896 [9], which was used in our experiments, is associated with the induction of inductive luciferase. However, the physiological roll of luminescence has not been clarified.

In this paper we show the characteristics of photon statistics in the early stages of cell proliferation of luminous bacteria under synchronous culturing.

II. EXPERIMENTAL PROCEDURES

We developed a measurement and analytical technique for the ultraweak photon emission field under a nonstationary transitional process. Measurements were performed with the single photoelectron counting technique, using a photomultiplier tube (PMT) based on a continuous and sequential measurement of the time intervals between two successive photoelectron pulses [10,11]. We calculated the Fano factor [$F_n(T)$; variance over mean of the number of photoelectrons observed within observation time T] as an indicator for photon counting statistics, which quantifies the deviation from

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Poisson statistics as a function of observation time T. For data processing, corrections are necessary for correlative components intrinsically accompanying the PMT dark pulses that are caused by cosmic-ray-induced photoelectron clusters [12] and microdischarges occurring in the PMT [13]. Because the afterpulse effects initiated by the incident photons are negligible under our conditions, we found that the photoelectron pulse events originating from incident light and the correlative components of dark pulses are statistically independent. Consequently, the Fano factor of incident light is deduced by subtracting the variance of the dark component, which had previously been measured, from that of the observed data.

The time course of intensity variation during cell proliferation can be expressed as an exponential function. The variance of the exponentially transient process is calculated from the following equations by using the intensity function of time, $I(t)=a\ \exp(bt)$, under the condition of observation time $T \ll 1/b$, where a and b are constants. When the number of photoelectrons and dark events from the PMT in the t_ith time period with observation time T are represented by $n_s(t_i,T)$ and $n_d(t_i,T)$, respectively, and their averages per unit time are indicated as $\mu_s(t)$ and μ_d, and average of total events as $\mu(t)$, they are

$$\mu_s(t)=\langle n_s(t,T)\rangle/T=ae^{bt}, \tag{1}$$

$$\mu_d=\langle n_d\rangle/T, \tag{2}$$

$$\mu(t)=\mu_s(t)+\mu_d, \tag{3}$$

where $\langle\ \rangle$ denotes an ensemble average. And we defined the variance of photocounts n through total measurement time T_N, represented as $\langle \Delta n^2\rangle$, as

$$\langle \Delta n^2\rangle=\frac{1}{T_n}\int_0^{T_N}[n(t,T)-\mu(t)T]^2dt. \tag{4}$$

From the independence of events (i.e., the incident light and dark components) $\langle \Delta n^2\rangle$ is expressed, using variances $\langle \Delta n_s^2\rangle$ and $\langle \Delta n_d^2\rangle$, as

$$\langle \Delta n^2\rangle=\langle \Delta n_s^2\rangle+\langle \Delta n_d^2\rangle. \tag{5}$$

Consequently, the Fano factor of the optical field is derived by

$$F_s(T)=\frac{\langle \Delta n^2\rangle-\langle \Delta n_d^2\rangle}{\dfrac{1}{T_N}\displaystyle\int_0^{T_N}\mu_s(t)T\ dt}=\frac{\langle \Delta n^2\rangle-\langle \Delta n_d^2\rangle}{\dfrac{a}{T_Nb}\,(e^{bT_N}-1)}. \tag{6}$$

To verify the performance of our experimental system and use it as a reference for the measurement of bacterial bioluminescence, we analyzed the statistical nature of light emission from a light-emitting diode (LED) (model TLRH 180P; Toshiba, Japan, with 50 dB optical attenuators) that was controlled by a standard current generator and a microcomputer to generate $\mu_s=a\ \exp(bt)$, where $a=74.2$ counts/s and $b=7.9\times10^{-4}\ s^{-1}$. The LED source operated under these conditions was found to exhibit a Poisson distribution with a Fano factor accuracy of 1.0 ± 0.003 through observation times of 0.1–5 ms as shown in Fig. 1.

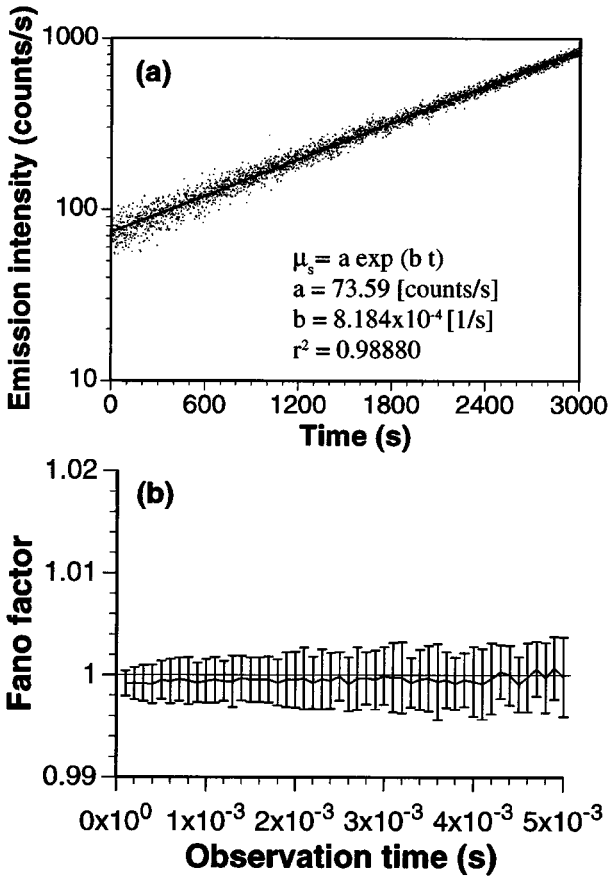

FIG. 1. (a) A result of the time course of photon emission intensity determined from a LED driven by a computer-controlled current generator to exhibit the exponential increase in intensity with time. The equation in the inset is an exponential function obtained from a least-squares curve fitting and the square of the correlation coefficient (r^2). (b) Fano factor vs observation time characteristics of LED photon emission intensities shown in (a). Error bars represent standard deviations of 10 experimental sets of data under the same condition.

III. RESULTS

Figure 2(a) shows the result of intensity analysis in the time course of bacterial photon emission from the lag phase to the proliferating log phase under liquid culturing [14,15]. A single cell was isolated by diluting a cell suspension. After preparing 10 samples (in 2 ml quartz vials) that resulted in diluting the cell density to 0.2 cells/2 ml, we selected one sample that was expected to contain a single cell by measuring the emission intensities. The intensity of a single cell in the early lag phase is estimated to be approximately 3.5×10^3 photons/s, which accounted for the total efficiency of detection. After a gradual decrease in emission intensity and prolonged lag phase ($\sim$5 h), an exponential increase in photon emission (log phase) was observed. We analyzed 3 parts of cell growth periods shown as I, II, and III in Fig. 2(a). To obtain adequate numbers for statistical accuracy in our analysis, we established the total number of photoelectron pulses to be 1.2×10^6. In the first period, the number of

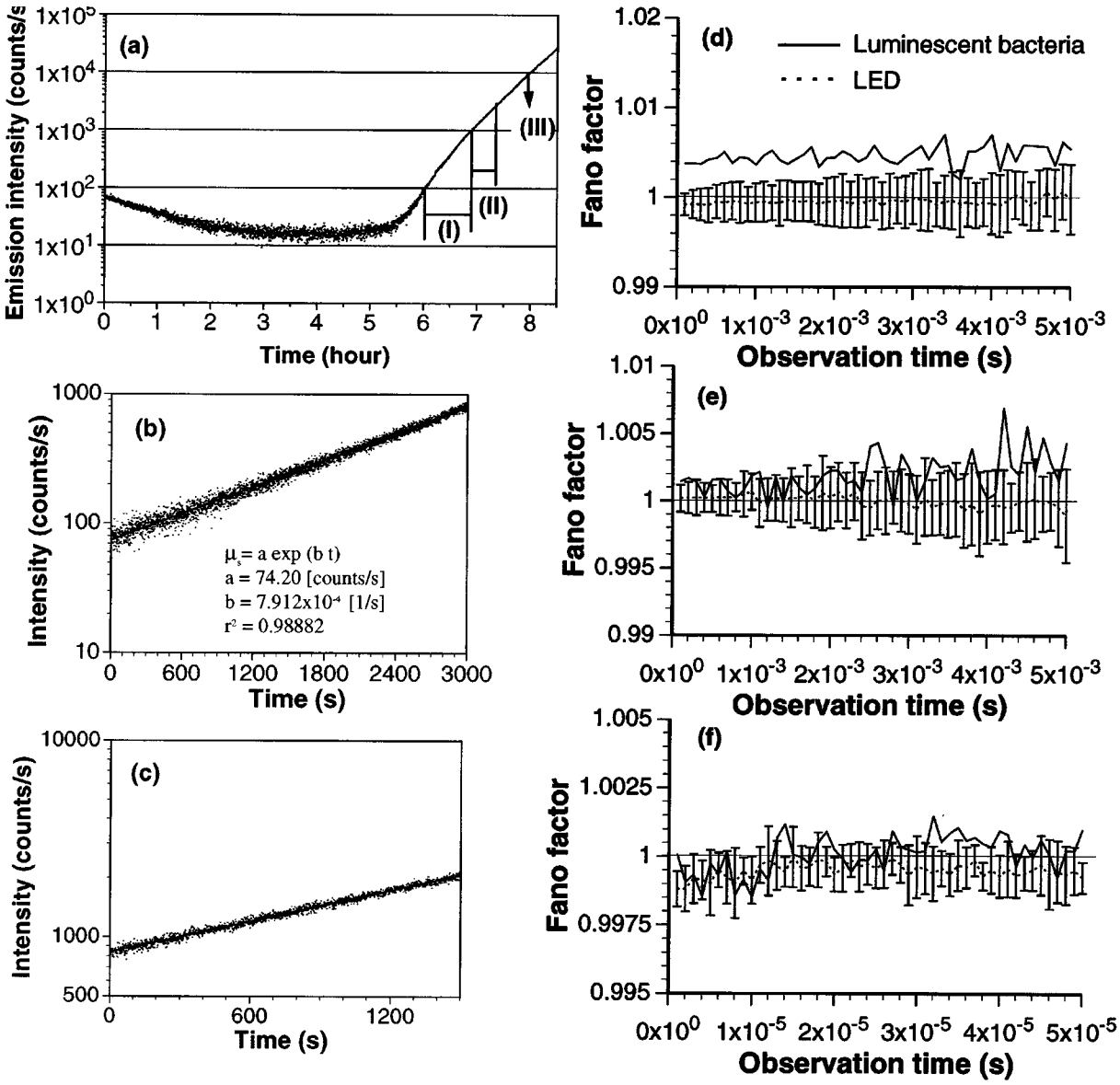

FIG. 2. (a) A typical result of measuring the time course of photon emission intensity of *Photobacterium phosphoreum* IFO 13896 from the lag phase to proliferating log phase under synchronous culturing. (b), (c) Time course of emission intensity corresponding to time regions I and II in (a), respectively. Solid lines are fitted curve using the equation inserted in (b). (d)–(f) Fano factor vs observation time characteristics for periods I, II, and III in (a), and a comparison with that of a LED (dotted line) driven to exhibit the same emitted intensity function as the *Photobacterium phosphoreum* shown in (b), (c). Error bars represent standard deviations of 10 experimental sets of LED data.

cells was roughly estimated to be 5–50. However, the light intensity per cell mass has been reported to increase linearly at a rate of 1.5 times per generation time; and this is explained as continuous activation or induction of the luciferase system during cell growth [16]. In our study therefore, the real number of cells should also be slightly lower than the estimated numbers. As shown in Fig. 2(b), the time course data of light intensity matched the exponential function sufficiently with a correlation coefficient of $r^2 = 0.988\,82$. Calculated Fano factors versus observation time at defined regions are shown in Figs. 2(d)–2(f), where they are compared with the Fano factors that were obtained from the LED with similar exponential time courses of bacterial light emission. The results indicate that during the primary stage of cell proliferation, the photon statistics show super-Poisson behavior, which changes to Poisson statistics according to the increase in the number of cells [Figs. 2(e) and 2(f)]. Averaged data obtained from three independent measurements in the intensity range of $10^2 - 10^3$ counts/s corresponding to Fig. 2(d) are displayed in Fig. 3.

IV. DISCUSSION

Super-Poisson statistics for the photon counting distribution are interpreted by the clustering of excitation and emission processes [17], where the optical field is composed of a sequence of independent flashes initiated by Poisson random time events. Observation time characteristics of the Fano factor imply photon correlation properties. The Fano factor is described by the following equation, using the degree of

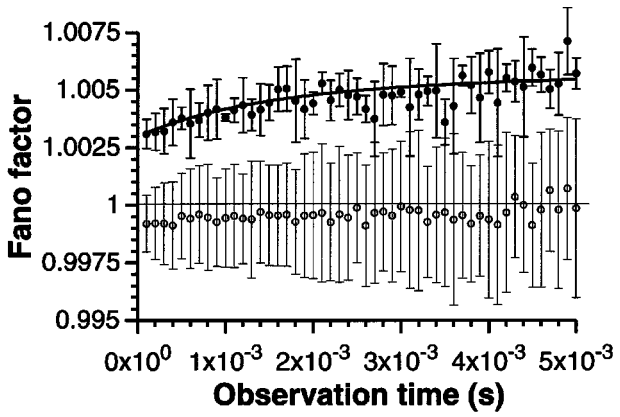

FIG. 3. Fano factor vs observation time characteristics of early stage emission [corresponding to the region of I in Fig. 2(a)] with the proliferating log phase of *Photobacterium phosphoreum* (filled circles) obtained from three independent measurements in comparison with a LED (open circles), and the theoretical curve fitted by the analysis. This fitted function is shown in Eqs. (7)–(10).

freedom parameter M, M_p, M_m, and multiplication factor of α [17],

$$F_n(T)=1+\frac{\langle n\rangle}{M}+\langle\alpha\rangle\left\{\frac{1}{M_p}+\frac{1}{M_m}\right\} \tag{7}$$

with the assumption that each flash event is a realization of a time-decaying chaotic optical field. When the spectrum is assumed to be Lorentzian, the experimental result can be theoretically analyzed, using Eq. (7) with the following parameters [17],

$$M=\frac{2\theta^2}{e^{-2\theta}+2\theta-1}\quad(\theta=T/\tau_c), \tag{8}$$

$$M_p=\frac{2\beta}{e^{-2\beta}+2\beta-1}\quad(\beta=T/\tau_p), \tag{9}$$

$$M_m=\frac{2\theta}{e^{-2\theta}+2\theta-1}. \tag{10}$$

Here, τ_p is the characteristic decay time of the individual flash event of excitation, and $1/\tau_c$ denotes spectral bandwidth of the emission. From the theoretical curve shown in Fig. 3, $\tau_p=1.8$ ms and $\alpha=0.003$ were estimated.

In view of the relationship between excitation events and emission events, photon emission can be represented as a single emission accompanying random deletion, which is determined by the efficiency (η) of excitation and emission process [18]. Then the Fano factor of emission events $F_n(T)$ is expressed by using that of excitation events $F_m(T)$ under the condition that the characteristic time of the excitation (τ_e) is restricted as $T\gg\tau_e$.

$$F_n-1=\eta(F_m-1). \tag{11}$$

The total efficiency of photon detection, in view of the quantum yield of the light emission ($\sim$10%), is approximately 0.15%. Hence the multiplication factor of an excitation event is deduced to be 2.0.

Although biological mechanisms to interpret our results are not known at present, the origin of the photon emission clustering process seems to be associated with regulation mechanisms within the reaction circuitry related to production and activation of luminescent enzymes (luciferase and/or aldehyde-synthesis enzymes), which is characterized by inducible luciferase syntheses. Specifically, it is deduced that abrupt generations of excited intermediators that are derived by the luciferase synthesis in the early stage of induction (enzymatic activity is not sufficient) is implicated with the clustering.

V. CONCLUSION

We demonstrate that the statistics governing endogenous biological processes could in principle be accessed through the statistical nature of emitted photon fields so that physiological information on biological self-regulation eventually manifesting as biological order or disorder may be derived. Meanwhile statistical analysis has potential usefulness not only for bioluminescence, but also for the general photon emission phenomena of living organisms (biophoton emission) generated from ordinary biological metabolic system [3,4,19], and quantum statistical nature of biological photon fields could provide a novel expression of the function of organisms.

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ORTEC, U.S.), and a pulse interval counter (Tohoku Electronic Industrial Co., Sendai, Japan), which records pulse-to-pulse interval consecutively by clock (80 MHz) pulse counting, using 4 cascaded counters (27 bit) to prevent missing pulses, which are provided from the discriminator as the minimum pulse pair with a resolution of 50 ns. Dead time of the system is determined by a constant fraction discriminator as 50 ns (nonparalyzable), and maximum count rate is 3×10^4 counts/s, limited by the performance of data transfer from an interval counter to a storage device. Time resolution is 12.5 ns and the capacity for the number of pulses measured is 2.7×10^8 events. The PMT was cooled at -40 ± 0.1 °C using a pulse tube cryocooler (Cryo-Mini; Iwatani gas Co., Tokyo, Japan) incorporated in a vacuum chamber to prevent dark counts drift. The number of dark counts from the PMT is 14.15 counts/s. Quantum efficiency of the PMT is 5% at 490 nm and the active surface diameter is 46 mm with a spectral sensitivity from 400 to 900 nm. Light collection efficiency is 40% and no lenses were used. A work station (SUN 4; Sun Microsystems) was used for the analyses after data acquisition.

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