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Unstably stratified cultures of luminous cells

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Dense and open to air cultures of *luxCDABE*-gene fused *Ralstonia eutropha* in a cylindrical vessel exhibit bioconvection, which accounts for fluctuating bioluminescence. The characteristic period of fluctuations is ~10 min, when an unstable oxic–anoxic interface develops in close proximity (~1 mm) to the meniscus. Formation of a particular interface in the deeper layers (~2–3 mm) results in a noise which is close to Brownian.

Key words: bioconvection, bioluminescence, fluctuations, *lux*-genes, oscillations

INTRODUCTION

The most important factor determining the development of naturally occurring gradient systems is the presence or absence of oxygen [1]. The steepness of the oxygen gradient depends on the amount of oxygen-consuming organic matter. Fast depletion of oxygen accounts for stratification of dense bacterial suspensions into oxic and anoxic layers [2–4]. Vertically stratified bacterial cultures represent unstable distribution of mass in fluid, called inversion [5]. Inversions do collapse in a fairly controlled way that generates the bioconvection cells [2, 5]. In the present work we report unstable bioluminescence signals from inverted cultures of *luxCDABE*-gene fused *Ralstonia eutropha* and interpret them in terms of bioconvection.

MATERIALS AND METHODS

Cultures

Ralstonia eutropha AE1239, harbouring the *luxCDABE* genes of *Vibrio fisheri* inserted under the control of a copper-inducible promoter [6], was grown in LB containing 20 μg tetracycline hydrochloride (Tc) / ml to prevent the cells from losing their plasmid. The LB consisted of 10 g tryptone (Oxoid), 5 g yeast extract (Oxoid) and 10 g NaCl (Lachema) in 1 l distilled water. To start the growth, 1 ml overnight stock was added to 100 ml LB + Tc medium. Cultures were shaken at 30 °C in Erlenmeyer flasks with wool cotton stoppers. Bacteria were grown to mid-log phase (OD_{600} = 0.3, biomass = 0.15 mg ml^{-1}).

Bioluminescence assays

The induction of the culture preincubated in a closed vial at 4 °C for 18 h (*A*) or a freshly harvested culture (*B*) was started by adding $CuSO_4$ to give 2 mM, the culture (10 ml) was then stirred at 22 °C with a magnetic bar (20 × 5 mm) at 160 rpm for ~7 (*A*) or ~3 h (*B*). Samples (0.6 ml) were put into glass test-tubes (8.9 i.d. × 45.2 mm). During the assays the samples were not stirred and maintained in a luminometer cell at 18 °C. An experimental setup of the integrated whole cell biosensor – fiber optic system was described previously [6]. Specifically, a homemade fiber optic luminometer was used to monitor bioluminescence [8]. The luminometer consisted of a temperature-controlled cell unit, photomultiplier (FEU-115, Russia) working in photon counting mode, controller, PC and fiber bundle (fused quartz, ∅ 8 mm), which was perpendicularly directed to the axis of the cylindrical sample. The average values of data points corresponding to 60 s of recordings were calculated and used for the presentation of data in 1000 counts per second (kcps) units. The fluctuating signals were analyzed in the same manner as described previously [7, 8].

RESULTS AND DISCUSSION

The transition of culture *A* from stirred to unstirred conditions resulted in complicated signals shown in Fig. 1. The observed step-like patterns occur due to stratification of the water (culture) column into oxic (luminous) and anoxic (dark) layers. Such adaptive responses to static conditions [4] supposedly indicate also an inversion of bacterial culture [5].

The corresponding spectra of bioluminescence intensity fluctuations are shown in Fig. 2. The closeness of the periods of dominant peaks to the duration of inversion could be noticed as an essential semi-qualitative result of presented data. Both temporal parameters can be associated with the same time-scale process – diffusive penetration of oxygen from the air–water interface to the developing oxic–anoxic interface level in the water (culture) column.

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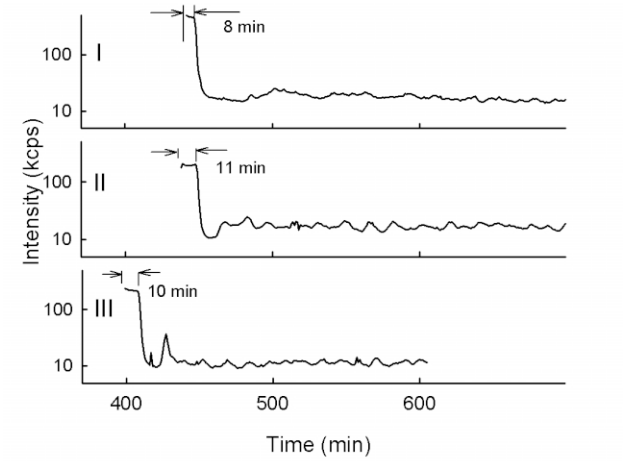

Fig. 1. Dependences of light emission from cultures *A* on time (I, II and III – records of three experiments carried out monthly). The starting points of each curve indicate the moment of sampling

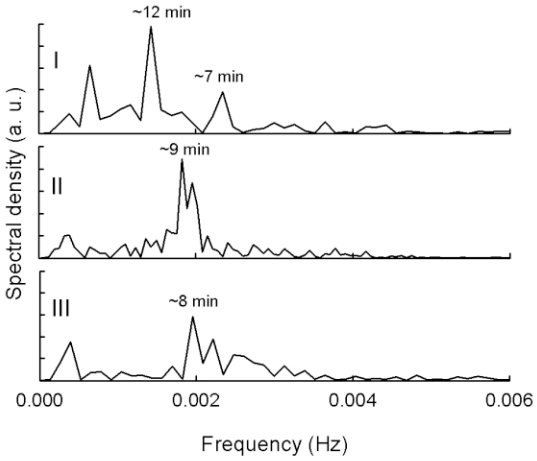

Fig. 2. Spectra of bioluminescence intensity fluctuations in cultures *A* (see Fig. 1). The corresponding periods in minutes are shown above spectral peaks

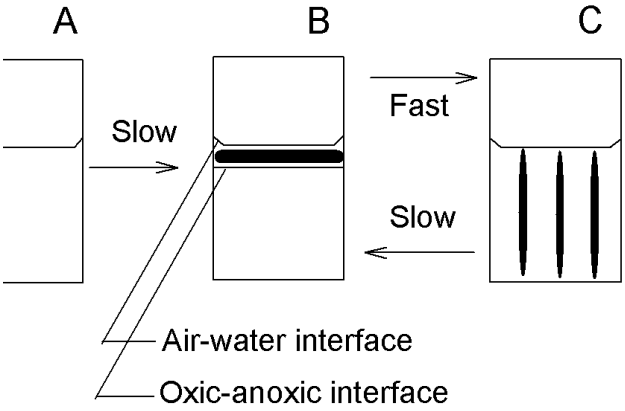

Fig. 3. Schematic presentation of bioconvection-exhibiting culture. Overbalanced density regions are in black

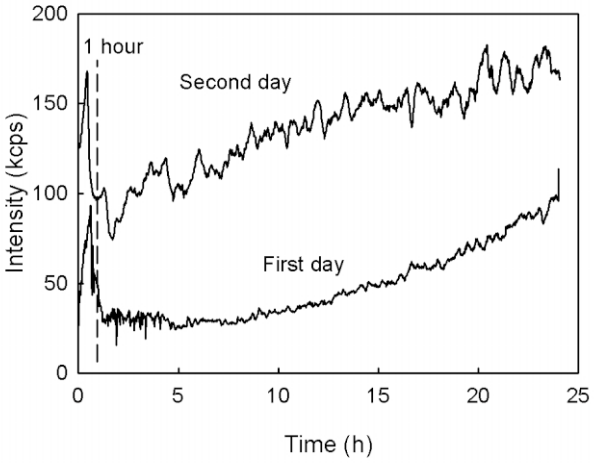

Fig. 4. Kinetic behavior of light emission from *R. eutropha* (culture *B*) just after sampling and after short-lasting shaking (~10 s) of the same sample next day

The time needed for the oxygen to diffuse over the oxic layer is $t_D = L^2/D$, where L is the characteristic thickness of the layer and $D = 2.12 \times 10^{-5}$ cm^2/s is the diffusion coefficient of oxygen in water. Assuming the equality of t_D to the experimentally obtained time scale (7–12 min) one can estimate L ~0.9–1.2 mm. The instability of a thin oxic layer (~1 mm) seems to be the reason for oscillatory bioluminescence exhibiting spectra with a small number of dominant frequencies (Fig. 2).

The processes in an unstably stratified culture are sketched in Fig. 3. The continuous upward swimming of cells results in a density inversion, *i.e.* the surface layer of the suspension becomes denser than the bulk (transition A–B). This configuration is mechanically unstable, and through a Rayleigh–Taylor instability the top layer sinks in vertical plumes (B–C) [2]. The sustained circulation of biomass (B–C–B–…) inevitably perturbs the availability of oxygen to its consumers and modulates oxygen-sensitive bioluminescence. Therefore, fluctuations of bioluminescence are an indirect measure of hydro-mechanical circulations and consequent respiratory rhythms in the culture. The closeness of the characteristic period of fluctuations to the duration of stratification (inversion) (Fig. 1, 2) indicates that convective self-mixing (transition B–C) is significantly faster than the aerotaxis-assisted inversion of the mechanically mixed or "self-mixed" bacterial culture (transitions A–B and C–B). The previously reported oscillations with the period of ~10 min in standing cultures of *lux*-gene fused *Ralstonia eutropha* [9] and *Escherichia coli* [8] can be interpreted in exactly the same manner, *i.e.* assuming bioconvection or circulatory-respiratory rhythms in a culture. Interestingly, in the previous papers on unstable bioluminescence, the high frequency oscillation (~10 min) was attributed to intra-cellular 'respiratory' rhythms [7–9]. However, the mechanism of synchronization of cellular processes in the culture has not been recognized. The currently suggested interpretation of the experimental results in terms of bioconvection gives us a reasonable

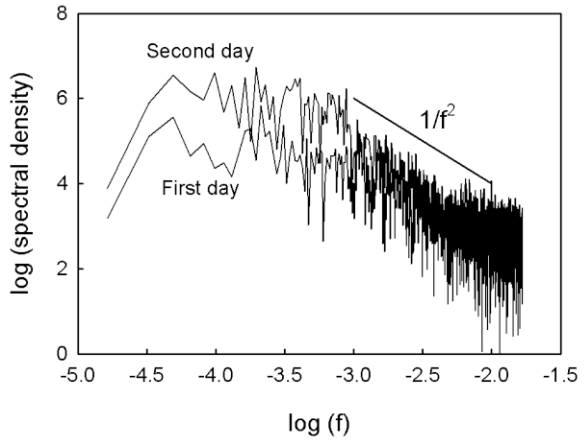

Fig. 5. Spectra of bioluminescence intensity fluctuations in culture *B* observed the first and the second day (see Fig. 4)

explanation of the synchronization. Seemingly, the synchronous behavior of cells may be achieved via global hydro-mechanical processes, which occur in the open to air suspensions of the heavier than water swimming bacteria (Fig. 3). Because of this interpretation, we guess that appropriate bioluminescence oscillations could not be observed, for example, in the suspensions of non-motile cells or in zero gravity systems.

The *B* cultures exhibit a longer adaptation to static condition (t_D ~40–60 min, *L* ~2–3 mm) and noisy bioluminescence (Fig. 4). The spectra of the observed fluctuations in log-log scale are shown in Fig. 5. Cultures produce a noise that is close to Brownian ($1/f^2$), resembling Brownian motion of a particle in one dimension [10]. One can suggest that the character of the observed fluctuations of bioluminescence intensity is dependent on the steepness of gradients in the culture and a corresponding location of the oxic–anoxic interface. The relatively low gradients of oxygen and density in culture *B* may account for a fuzzy boundary between the developing oxic and anoxic layers. In this case, the swimming rates and swimming directions chosen by bacteria can fluctuate in a broad range. The net result of such disordered bioconvection is noisy bioluminescence without any dominant frequency. Physically, bioconvective cultures seem to be similar to non-biological convective systems, which can also exhibit low-frequency noise and/or low-frequency oscillations [11].

In conclusion, data on fluctuating / oscillatory bioluminescence can be interpreted in terms of bioconvection. According to this interpretation, cells in the culture produce bioluminescence that mimics the action of the self-created "circulatory-respiratory system" [5] in the broth. Formation of unstable structures such as inversions or convective plumes can be regarded as a prime reason for noise in *lux*-gene based whole-cell biosensors.

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NESTABILIAI STRATIFIKUOTOS ÐVYTINÈIØ LÀSTELIØ KULTÛROS

S a n t r a u k a

Orui atvirose cilindrinëse kiuvetëse, uþpildytose *luxCDABE* genais modifikuotomis *Ralstonia eutropha* kultûromis, vyksta biokonvekcija, kuri nulemia bioluminescencijos fliuktuacijas. Charakteringas bioliuminescencijos svyravimø periodas yra apie 10 min, kai nestabilus skiriamasis paviršius tarp deguoná turinèio ir bedeguonio sluoksnio formuojasi arti menisko (~1 mm). Atitinkamo skiriamojo paviršiaus formavimasis gilesniuose sluoksniuose (~2–3 mm) sàlygoja bioliuminescencijos triukšmà, kurio spektras yra artimas Brauno triukšmo spektrui.