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Rhythmic gene expression in a purple photosynthetic bacterium, *Rhodobacter sphaeroides*

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Abstract Circadian rhythms are known to exist in all groups of eukaryotic organisms as well as oxygenic photosynthetic bacteria, cyanobacteria. However, little information is available regarding the existence of rhythmic behaviors in prokaryotes other than cyanobacteria. Here we report biological rhythms of gene expression in a purple bacterium *Rhodobacter sphaeroides* by using a luciferase reporter gene system. Self-bioluminescent strains of *Rb. sphaeroides* were constructed, which produced a bacterial luciferase and its substrate, a long chain fatty aldehyde, to sustain the luminescence reaction. After being subjected to a temperature or light entrainment regime, the reporter strains with the luciferase genes driven by an upstream endogenous promoter expressed self-sustained rhythmicity in the constant free-running period. The rhythms were controlled by oxygen and exhibited a circadian period of 20.5 h under aerobic conditions and an ultradian period of 10.6–12.7 h under anaerobic conditions. The data suggest a novel endogenous oscillation mechanism in purple photosynthetic bacteria. Elucidation of the clock-like behavior in purple bacteria has implications in understanding the origin and evolution of circadian rhythms.

Keywords: Bioluminescence; Luciferase reporter; Circadian rhythms; Evolution of biological clocks

1. Introduction

Circadian rhythms are an endogenous biological control mechanism that exhibits self-sustained oscillations with a period close to 24 h. This mechanism plays a central role in controlling a wide array of biological behaviors and has been shown to exist in all groups of eukaryotic organisms and oxygenic photosynthetic bacteria, cyanobacteria (see reviews [1–3]). In cyanobacteria, the general gene expression is known to be circadian, which is controlled by the central clock genes, the *kai* genes [4,5]. These genes encode a set of interacting proteins KaiA, KaiB and KaiC, which oscillate through a feedback control mechanism that transduces signals to regulate the downstream gene expression.

Circadian rhythms have not been documented in other groups of prokaryotes. However, homologs of certain *kai* genes appear to be ubiquitous among prokaryotic organisms. Recent evolutionary analysis has revealed the presence of conserved homologs of KaiB and KaiC in a wide range of prokaryotic genomes including those from purple photosynthetic bacteria [6,7]. Though little experimental evidence is available that has proved the existence of biological clocks in these organisms, it seems reasonable to postulate that anoxygenic photosynthetic bacteria may have a similar time keeping mechanism to that in cyanobacteria. Like cyanobacteria, purple bacteria perform photosynthesis which depends on light which alternates in cycles according to daily cycles. The long course of evolution may have thus allowed them to develop a mechanism that is able to anticipate day and night. The discovery of Kai homologs in purple photosynthetic bacteria has made these organisms more attractive for the study of the presence of circadian rhythms.

To date, there is only one report on rhythmic behaviors in anoxygenic photosynthetic bacteria. Van Praag et al. [8] showed that the activity of uptake hydrogenase in the purple bacterium *Rhodospirillum rubrum*, without entrainment, exhibited a 12 h ultradian oscillation cycle under a continuous light and warm temperature condition (32 °C) but switched to a 24 h circadian cycle in continuous darkness but at a lowered temperature (16 °C). Though the experiment is intriguing, it is not clear whether the general gene expression in this organism is rhythmically controlled. Questions also remain on whether the rhythms can be self-sustained or properly entrained.

In the present study, we chose a purple photosynthetic bacterium, *Rhodobacter sphaeroides*, which has a well-defined system for genetic manipulations, to investigate the possible rhythmic behavior. We constructed luciferase reporter bacterial strains that generated luminescence that could be used to monitor the oscillation patterns of gene expression in a constant condition after being entrained by temperature or light. The constructed *Rb. sphaeroides* strains were self-bioluminescent, which produced luciferase as well as the luciferase substrate, a long chain fatty aldehyde, intracellularly, to sustain the luminescence reaction. With the newly constructed reporter strains, we have shown that the expression of many genes in these organisms exhibited self-sustained rhythmicity, the period of which is modulated by the presence or absence of oxygen. Under aerobic conditions, circadian oscillations of gene expression were observed; under anaerobic conditions, ultradian oscillations were observed. The data suggest the presence of an endogenous clock mechanism that is different from that in cyanobacteria.

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2. Materials and methods

2.1. Bacterial strains and culture conditions

In this study, *Escherichia coli* strains were grown in a liquid or solid LB medium at 37 °C. *Rb. sphaeroides* wild type (strain 2.4.1) and engineered strains were grown with a tungsten light illumination at 30 °C before entrainment. The light and temperature conditions of entrainment are indicated in Section 3. Three different media were used to culture *Rb. sphaeroides*, the LB, the Sistrom [9] or the enriched Sistrom (Sistrom medium plus 0.05% casamino acids, 0.25% yeast extract and 0.25% tryptone) media. When necessary, antibiotics were added at the following concentrations: kanamycin, 50 μg/ml; streptomycin, 12.5 μg/ml. *Rb. sphaeroides* was grown either aerobically or anaerobically. The anaerobic condition was created using either a sealed anaerobic jar containing an AnaeroGen pouch (Oxoid Limited, Hampshire, England) or a COY Laboratories anaerobic chamber (Grass Lake, MI).

The *Rhodobacter* cultures were generally grown for 2 days at 30 °C after which the cells were assumed to be in the stationary phase. The wild type *Rb. sphaeroides* cells have a doubling time of ≈3 h either under aerobic or photoheterotrophic conditions [15], reaching the stationary phase in roughly 48 h. The cells were subsequently moved to lower temperatures (23 or 16 °C) and treated with a temperature or light entrainment regime of 1.5 days before bioluminescence was measured in the constant free-run condition.

2.2. Construction of self-bioluminescent Rhodobacter strains

Self-bioluminescent *Rb. sphaeroides* strains were created by introduction of the bacterial luciferase genes (*luxA* and *luxB*) and the aldehyde biosynthesis genes (*luxC*, *luxD* and *luxE*) producing the substrate for the luciferase. In this experiment, the *luxCDE* operon responsible for the aldehyde synthesis from *Photorhabdus luminescens* [10] was cloned into a derivative of the shuttle vector pJRD215 [11] through *Eco*RI digestion and subsequent ligation and transformation via electroporation. Since the original pJRD215 plasmid shares the same antibiotic resistance markers (Km^r and Sm^r) with the *luxAB*-containing transposon (Tn5) (Nm^r/Km^r and Sm^r, [12], see Fig. 1), to facilitate the subsequent selection against the *E. coli* DNA donor during conjugation, the kanamycin resistance cassette (Km^r) from pJRD215 was truncated by using an *Xho*I and *Sal*I restriction enzyme digestion followed by self ligation before the plasmid was used for subsequent transformation. The resultant pJRD::*luxCDE* construct was introduced into *Rb. sphaeroides* through triparental conjugation by spot-mating of *E. coli* donor and *Rb. sphaeroides* recipient cells [13]. During this process, the conjugal plasmid pRK2013 in *E. coli* was used as a helper [14]. The selection of exconjugants was performed by growing the cells anaerobically in the presence of streptomycin (Sm).

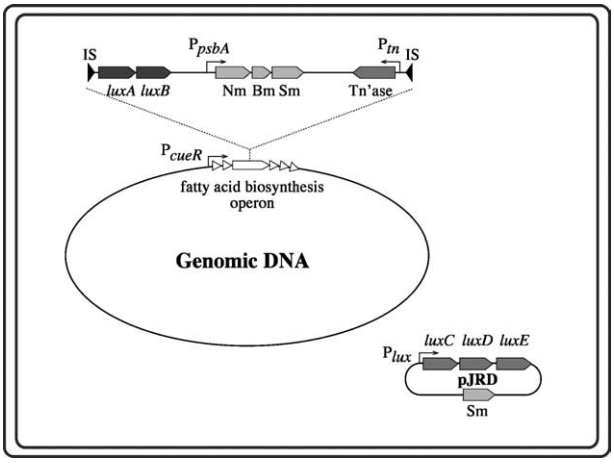

Fig. 1. Schematic diagram of a constructed self-bioluminescent *Rb. sphaeroides* strain (JX57) showing the genomic insertion site of the promoterless bacterial luciferase genes, *luxAB*. The *luxAB* genes were inserted into an operon for fatty acid biosynthesis, with the aid of a transposon Tn5 carrying a transposase gene (Tn'ase). In addition, the strain was made self-bioluminescent by introducing the genes (*luxCDE*) for the biosynthesis of the luciferase substrate, with the aid of a derivative of the shuttle vector pJRD215. The antibiotic resistance markers are indicated: Nm, neomycin; Bm, bleomycin; Sm, streptomycin. IS is the inverse sequence in the transposon. The Nm marker also renders kanamycin resistance. P*psbA*: promoter for the psbA gene from *Anabaena* sp.; P*tn*: promoter for the transposase gene; P*cueR*: promoter for the *cueR* gene in a fatty acid biosynthesis operon in *Rb. sphaeroides*; P*lux*: promoter for the *lux* operon originated from *P. luminescens*.

The selected exconjugants were further introduced with the bacterial luciferase genes (*luxA* and *luxB*). This was carried out with the aid of a plasmid pRL1063a containing the promoter-less *luxAB* genes from *Vibro fischeri* within a transposon Tn5 [12]. The plasmid was transferred to the *Rb. sphaeroides* strain containing *luxCDE* through triparental mating followed by random transposon insertion into the genome. The resulting conjugants were selected in an anaerobic condition in presence of kanamycin and streptomycin.

2.3. Bioluminescence assays and data analysis

Bioluminescence was measured at room temperature (23 °C) by a scintillation counter with coincidence disabled for photon counting (Beckman LS3801 liquid scintillation system). Manual sampling was carried out to monitor the rhythms of bioluminescence with each sample being measured three times to obtain the average of the measurement.

To measure bioluminescence from a solid culture after entrainment, a piece of agar with culture medium was laid at the bottom of a 20 ml scintillation vial, which was inoculated by colonies of the constructed reporter strains. The vial was kept in a closed jar with sterilized water at its bottom to keep the culture moisturized. The same vial was used for bioluminescence measurement throughout the experiment. To measure bioluminescence from a liquid culture grown under aerobic conditions, 300 μl of the liquid culture were transferred to a scintillation vial and bioluminescence was measured on the scintillation counter. For measuring bioluminescence from liquid cultures grown under anaerobic conditions, the cells were extracted from sealed anaerobic jars and exposed to air for 5 min. before measurement to provide oxygen for the luciferase reaction.

The raw data for the luminescence measurements were subjected to a Lomb–Scargle Fourier Transform analysis with the aid of the Autosignal™ program (Systat Software Inc. Point Richmond, CA). The method performs a least-square fitting of the original data with a sinusoidal parametric function [16]. Through regression at various frequencies, statistically significant components that are regularly oscillated can be extracted.

2.4. Inverse PCR

To determine the locations of *luxAB*::Tn5 insertion in the *Rb. sphaeroides* genome in a particular exconjugant strain, an inverse PCR strategy was applied. The procedure was modified from Ochman et al. [17]. Briefly, genomic DNA of the exconjugants of *Rb. sphaeroides* was isolated from a liquid culture according to Pospiech and Neumann [18] and was treated with appropriate restriction enzymes. Digested DNA was purified using Qiaprep Miniprep spin columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified DNA was further self-ligated and subsequently purified before the PCR reactions. The inverse PCR primers were used to amplify sequences upstream of the *luxA* gene as well as downstream of the transposase gene (Fig. 1). The PCR products were purified and used for DNA sequencing reactions. DNA sequencing was performed with a fluorescent dye-labeled dideoxynucleotide sequencing reaction kit (Amersham, Piscataway, NJ).

3. Results and discussion

3.1. Selection of self-bioluminescent strains and determination of transposon insertion sites

In this study, we have constructed self-bioluminescent *Rb. sphaeroides* strains, which produced luciferase as well as the luciferase substrate to sustain the luminescence reaction over a relatively long period of time. The strains were tested for the production of luminescence, which was most likely the