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Searching for a circadian clock in *Rhodopseudomonas palustris* Strain TIE-1 by oxygen entrainment

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Abstract

Circadian clocks are the internal timing mechanisms that facilitate organisms to regulate their activities in diurnal cycles. Although circadian clocks are ubiquitous among eukaryotes, cyanobacteria are the only bacteria in which circadian clocks have been conclusively demonstrated. Bioinformatic studies suggest that homologs of cyanobacterial clock genes also exist in other bacteria and archaea. In particular, these homologs in purple nonsulfur bacteria share a high similarity with cyanobacteria. In this study, I report that in purple nonsulfur bacteria *Rhodopseudomonas palustris* Strain TIE-1, the nitrogen fixation exhibited self-sustained rhythmicity in 48 hours after the entrainment by oxygen. In contrast, when this strain was entrained by light, nitrogen fixation only exhibited random oscillations. These results implicate oxygen as a potentially new environmental cue to entrain circadian clocks, and suggest TIE-1as a potential new bacterial species with circadian rhythms.

Introduction

Circadian clocks are the internal timing mechanisms that allow organisms to anticipate daily changes in the environment. Circadian clocks are ubiquitous among eukaryotes, thus suggesting circadian clocks as an adaptation to the diurnal cycles of light and temperature driven by the rotation of the earth. In the domain of bacteria, however, cyanobacteria are the only phylum in which circadian clocks have been conclusively demonstrated. Before the discovery of circadian clocks in cyanobacteria, it was believed that bacteria and archaea were incapable of generating circadian rhythms due to the short doubling time and the lack of nuclear structure [1, 2]. Now that has the dogma been broken, researchers tend to ask another question: are there any other bacteria or archaea possessing circadian clocks besides cyanobacteria? Benefiting from the discovery of *kai* genes in *Synechococcus elongatus* PCC 7942 [3] and the fact that a great number of genomic sequences are available in public database, a bioinfomatic study revealed that homologs of *kaiBC* exist in proteobacteria and archeae. In particular, homologs in some purple nonsulfur bacteria (PNSB) share a high similarity with *kaiBC* in the cyanobacterium *synechocystis*, which provided the possibility that PNSB are able to generate circadian rhythms [4]. Indeed, one of the PNSB, *Rhodospirillum rubrum*, has already been studied for its rhythmic activity of uptake hydrogenase before this bioinformatic study. However, the rhythms showed in this paper could not satisfy the fundamental properties of circadian rhythms due to the short period (~12h) and the lack of temperature compensation [5]. Recently, gene expression of another PNSB, *Rhodobacter sphaeroides*, is studied by introducing a bioluminescence reporter to this strain. Although a promising rhythm was reported in this paper, it was still not temperature-compensated [6].

The fact that PNSB are closely related to *Synechocystis* on the phylogenetic tree of *kaiBC* is not surprising since they are also photosynthetic bacteria. However, PNSB are anoxygenic phototrophs which use different electron donors other than water. Oxygen inhibits the growth and many other activities of PNSB including photosynthesis and nitrogen fixation. In the absence of oxygen, PNSB can grow photoautotrophically by using CO_2 as the carbon source or grow photoheterotrophically by using organic carbon sources. When they are exposed to oxygen, they can rapidly shut down photosynthetic systems and switch their energy and carbon source to organic compound (chemoheterotrophy) [7]. As early as in 1957, Cohen-Bazire et al. have shown that oxygen is the main environmental stimulus regulating the activity of PNSB [8].

Based the role of oxygen on the regulation of activities of PNSB, and the conservation of *kaiBC* genes between PNSB and cyanobacteria, I hypothesize that oxygen also plays a role on the activation of *kai* homologs in PNSB, and it can be an efficient environmental cue to entrain PNSB. If the hypothesis is true, rhythmic activities are expected to be observed after the entrainment of oxygen. *Rhodopseudomonas palustris* Strain TIE-1 was used to test this prediction in this project. This strain is isolated by Newman et al. at the iron-rich mat in Woods Hole, MA [9],and it is ideal for this study due to its fast growth and the presence of *kaiBC* homologs. The rates of nitrogen fixation were measured as the readout of activity of TIE-1. As an attempt to explore the role of oxygen on the circadian rhythms, I have shown that 1mM oxygen inhibited the growth and nitrogen fixation of TIE-1, and the nitrogen fixation exhibited self-sustained rhythmicity in 48 hours after the entrainment of oxygen. These results implicate oxygen as a potentially new environmental cue to entrain circadian clocks, and suggest TIE-1as a potential new bacterial species with circadian rhythms. If fully tested and confirmed, this study has significant impact on the mechanism as well as the evolution of circadian clocks.

Materials and Methods

Bacterial strain and culture conditions

The wild-type strain, *Rhodopseudomonas palustris* Strain TIE-1, was cultivated in freshwater-medium supplemented with sodium-acetate (25mM) as the carbon source and electron donor. Limited ammonia (0-1mM) was supplied in the medium in order to induce the nitrogenase. 50ml medium was used in each culture, while N_2 was supplemented in the headspace (110ml). To test the effects of oxygen stress, 10ml, 1ml and 0.1ml air was injected into anaerobic cultures. White fluorescence lights were used for the illumination. Growth of these cultures was monitored by increase of optical density at 600nm. The amount of bacterial chlorophyll a was measured by the absorbance of 805nm and 864nm.

Nitrogenase activity assay by gas-chromatography (GC)

Nitrogenase activity was measured by acetylene-reduction assay [10]. At each time point, 2ml aliquot was transferred to a N_2-filled bottle supplemented with a certain amount of air which keeps the cells at the original state. 10% gas in the bottle was replaced with the same amount of acetylene. Then bottles were incubated in the light at 30 ℃ from 1 hour to 6 hours.

Entrainments

The process of entrainments can be seen in figure 3. Three entrainment conditions were set up. For the entrainment by light (**Fig.3A**), cells were cultivated in 12-hours-light-and-12-hours-darkness cycles (LD) for 48 hours. After that, cultures were released to constant light condition (LL) and nitrogenase activities were measured every 6 hours. For the entrainment by O_2 (**Fig.3B**), cells were cultivated anaerobically for 12 hours and exposed to 2.1% O_2 for another 12 hours. This process was repeated once. Cultures were then released to anaerobic condition and nitrogenase activities were measured every 6 hours. The third entrainment (**Fig. 3C**) imitated natural condition. Cultured were exposed to light and 2.1% O_2 for 12 hours, then they were transferred to darkness and anaerobic condition for another 12 hours. This process was repeated once. After that cultures were released to constant darkness and 0.21% O_2 condition. As entrainment A and B, nitrogenase activities were measured every 6 hours.

Results and Discussion

The effect of oxygen on the growth and nitrogen fixation of TIE-1

Before entraining by oxygen, it is critical to test how much oxygen is appropriate for the entrainment and how cells response under different oxygen concentrations. If the concentration is too high, cells may stop growing or even initiate cell death. If the concentration is too low, the small amount of oxygen may be not sufficient for the entrainment. Therefore, it is necessary to test the effect of oxygen on the growth of TIE-1at different oxygen concentrations.

Four groups of cultures were set up in duplicates with oxygen at the concentration of 0, 1mM, 0.1mM and 0.01mM. As shown in **Fig. 1A**, comparing to cultures without oxygen, cultures with 1mM oxygen showed obvious differences in both turbidity and the amount of bacterial chlorophyll a, while cultures with 0.1mM and 0.01 mM oxygen only showed a subtle difference. These differences were quantified by growth curves and the curves of bacterial chlorophyll a (**Fig. 1BC**). Doubling time of these cultures is shown in **Table 1**. These results confirm that growth of TIE-1 is inhibited by oxygen, while the highest concentration in this experiment, 1mM, had the most obvious inhibitory effect. The doubling time was increased by 5 hours. 0.1mM and 0.01mM oxygen did not inhibit the growth as much as the 1mM and the doubling time was increased by 1 hour. The reason why 0.1mM and 0.01 mM oxygen showed the similar inhibitory strength is not clear. It is likely that the concentrations of dissolved oxygen in the medium were similar due to the small amount of oxygen in these two groups.

Except for growth and bacterial chlorophyll a, nitrogenase activities were also measured to assess the effect of oxygen. Compared to the negative control which 10mM ammonia was supplemented in the medium, there was almost no nitrogen fixation in cultures with 1mM oxygen (**Fig.2**). Nitrogenase activities in the two cultures with the oxygen at the concentration of 0.1mM showed a big variation which invalidated the result of this group. Interestingly, in the group where oxygen concentration was 0.01mM, the nitrogenase activities increased more than 30 folds after 2 hours (**Fig.2A**). It is likely that in the first 2 hours the nitrogenase activity was inhibited by oxygen, and the small amount of oxygen was depleted such that the nitrogenase was induced. But it is not clear why the nitrogenase activity was even higher than that in the group without oxygen after 6-hour incubation. The average of nitrogenase activity in 6 hours was calculated and plotted in **Fig. 2B.** Because of the variations among different incubation durations (0-2h vs. 2h-6h), it seemed that 6-hour incubation would be necessary to assess the nitrogenase activity.

Based on these results, I decided to use oxygen at the concentration of 1mM to entrain TIE-1 cultures.

Entrainments by different environmental cues

As shown in **Fig.3**, three entrainments were performed in this study. Light is the most common environmental cue utilized by circadian research. After entrained by light-dark cycles (LD), bioluminescence levels in *Rhodobacter sphaeroides* exhibited 12-hour oscillations [6]. Thus, LD cycles were used in this study to compare with the entrainment by oxygen (**Fig. 3AB**). As shown in **Fig. 3C**, the combination of light and oxygen was also used to imitate natural condition.

After entrained for two cycles (48 hours), cultures were released to constant conditions. Nitrogenase activities were measured to determine whether there was rhythmicity. Strikingly, both of the oxygen entrainment and the combination of light and oxygen rendered the

nitrogenase activity to be rhythmic. The free running period, which is the duration of each cycle under constant conditions, was roughly 20 hours (**Fig. 4BC**). As for the entrainment by LD cycles, the nitrogenase activity showed a peak in the first 12 hours and then another peak in the next 30 hours. This activity is more likely a random oscillation instead of circadian rhythms. Compared to this random oscillation, activities in **Fig.B** and **C** showed two even cycles which was one of the properties of circadian rhythms.

Even though the results here showed circadian-like rhythms, these rhythms need to be confirmed by temperature compensation which is another important property of circadian rhythms.

Conclusion

As an attempt to explore the role of oxygen on the circadian rhythms, I have shown that 1mM oxygen inhibited the growth and nitrogen fixation of TIE-1, and the nitrogen fixation exhibited self-sustained rhythmicity in 48 hours after the entrainment of oxygen.

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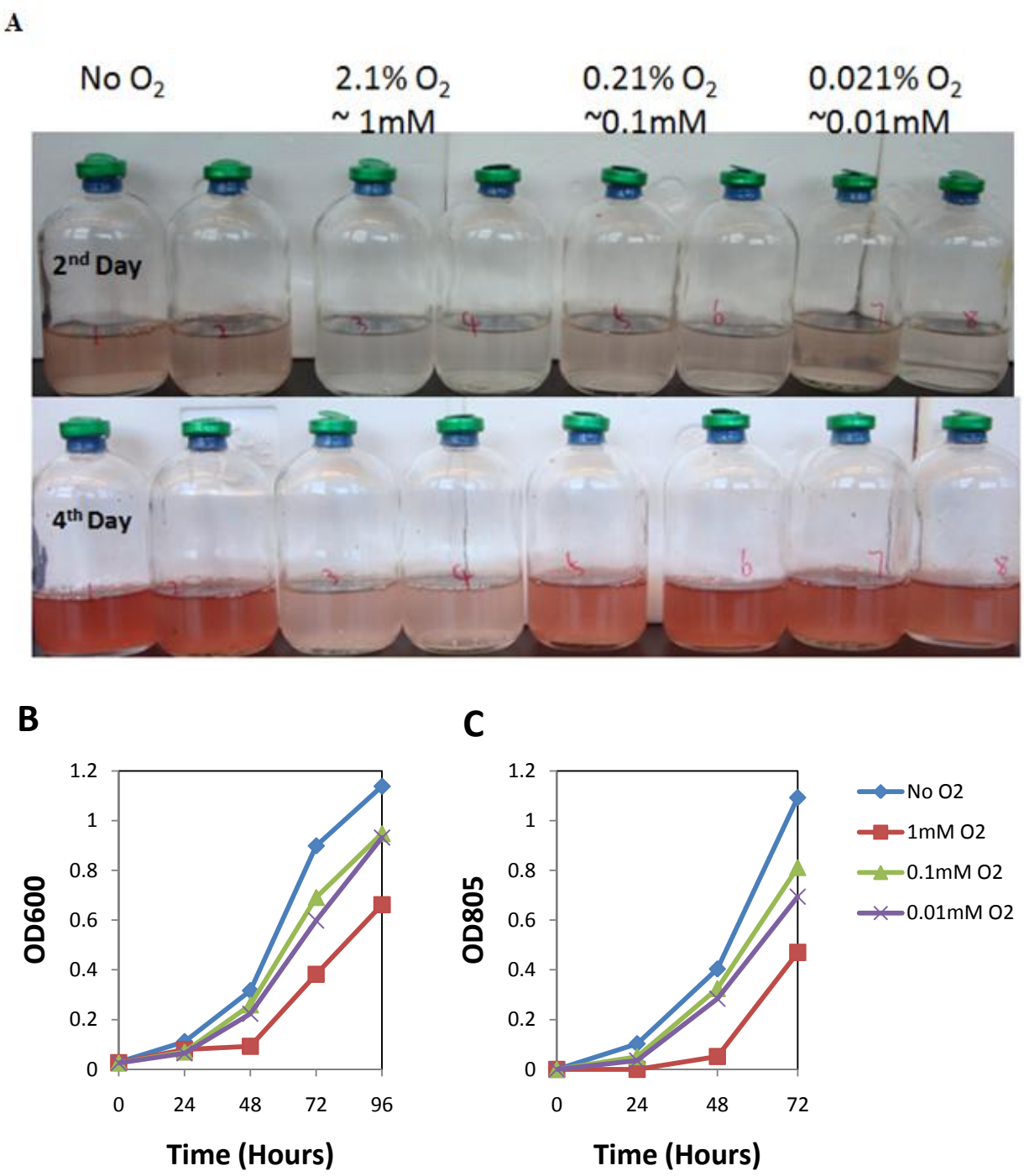

Figure 1. The effect of oxygen on the growth of TIE-1 strain. (A) Four groups of cultures were set up when the effect of oxygen was tested. Experiments were done in duplicates. In the first group, cells were cultivated anaerobically. In the other three groups, 10ml, 1ml and 0.1ml filtrated air was injected to the bottle to replace the same volume of nitrogen in the headspace. The concentrations of oxygen were roughly calculated by the percentages of air in bottles. 2.1%, 0.21% and 0.021% of oxygen roughly equal to 1mM, 0.1mM and 0.01mM oxygen in 100ml

headspace. (B) Growth curves measured by OD600. (C) The amount of bacterial chlorophyll a measured by OD805. Blue, cultures without oxygen; red, cultures with 1mM oxygen; green, cultures with 0.1mM oxygen; purple, cultures with 0.01mM oxygen.

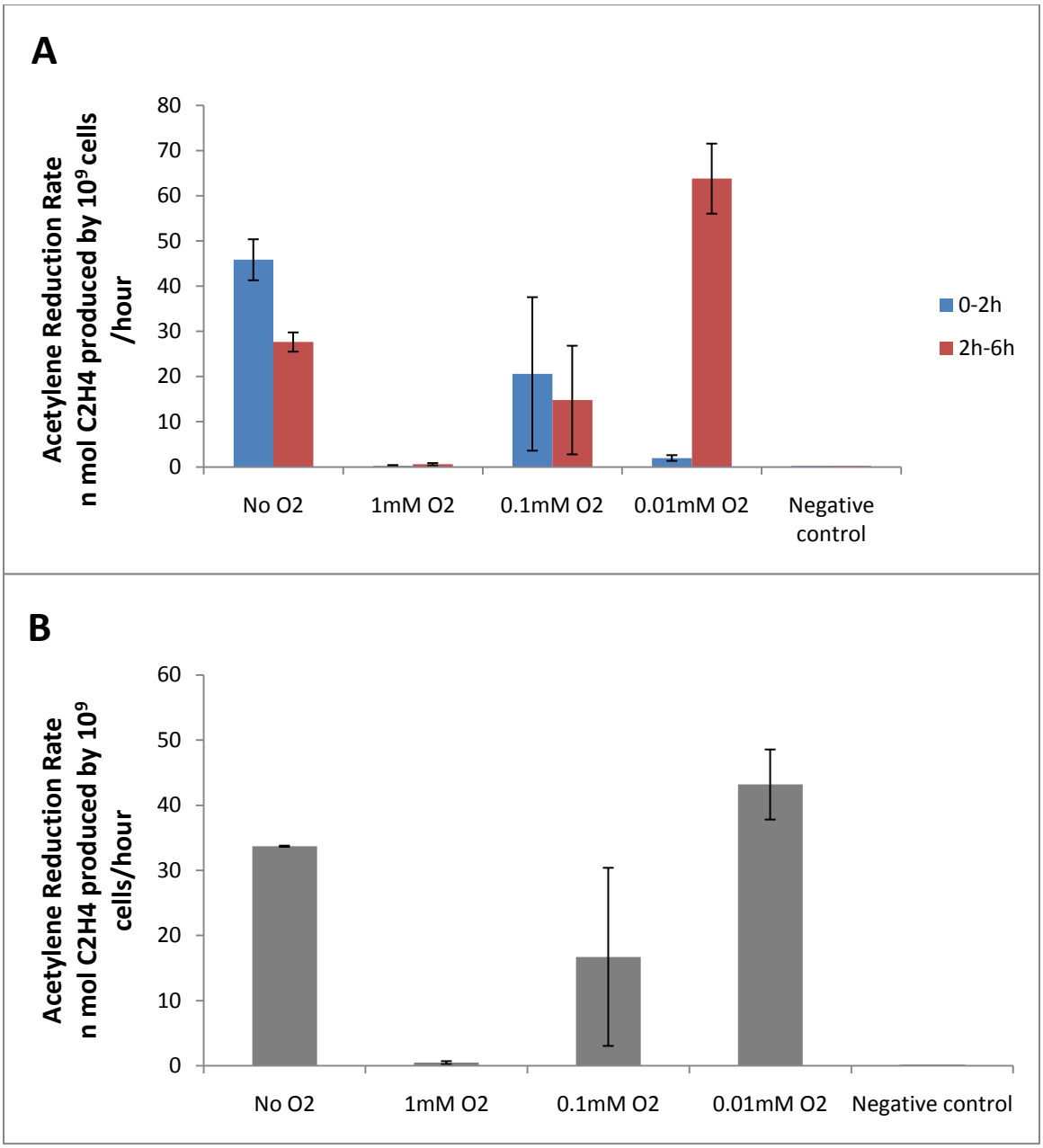

Figure 2. The effect of oxygen on nitrogenase activities. (A) Nitrogenase activities of cultures at different oxygen concentrations. Blue, nitrogenase activities measured in first 2 hours; red, nitrogenase activities measured in the next 4 hours (2h-6h). (B) Average nitrogenase activities in 6 hours.

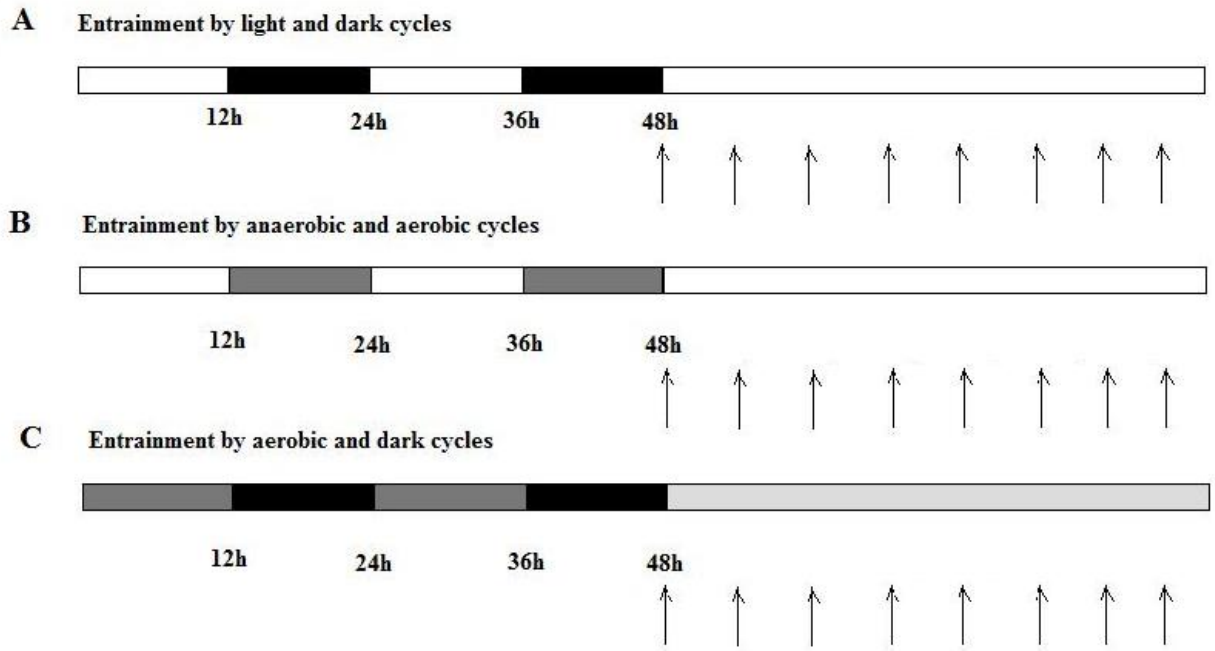

Figure 3. Entrainment setup. (A) TIE-1 cultures were entrained by light and dark cycles under anaerobic condition. White box and black box represent light and darkness conditions, respectively. (B) TIE-1 cultures were entrained by oxygen cycles under constant light condition. White box represents anaerobic condition, while dark gray box represents aerobic condition with 2.1% oxygen. In (A) and (B), the long-white box starting at 48 h represents constant light and anaerobic condition. (C) TIE-1 cultures were entrained by oxygen cycles and light-dark cycles. Gray box represents light and aerobic condition with 2.1% oxygen, while dark box represents darkness and anaerobic condition. The light gray box starting at 48 h represents constant light condition and supplemented with 0.21% oxygen in the headspace. Arrows represent the time points when nitrogenase activities were measured. There were three cultures in each entrainment.

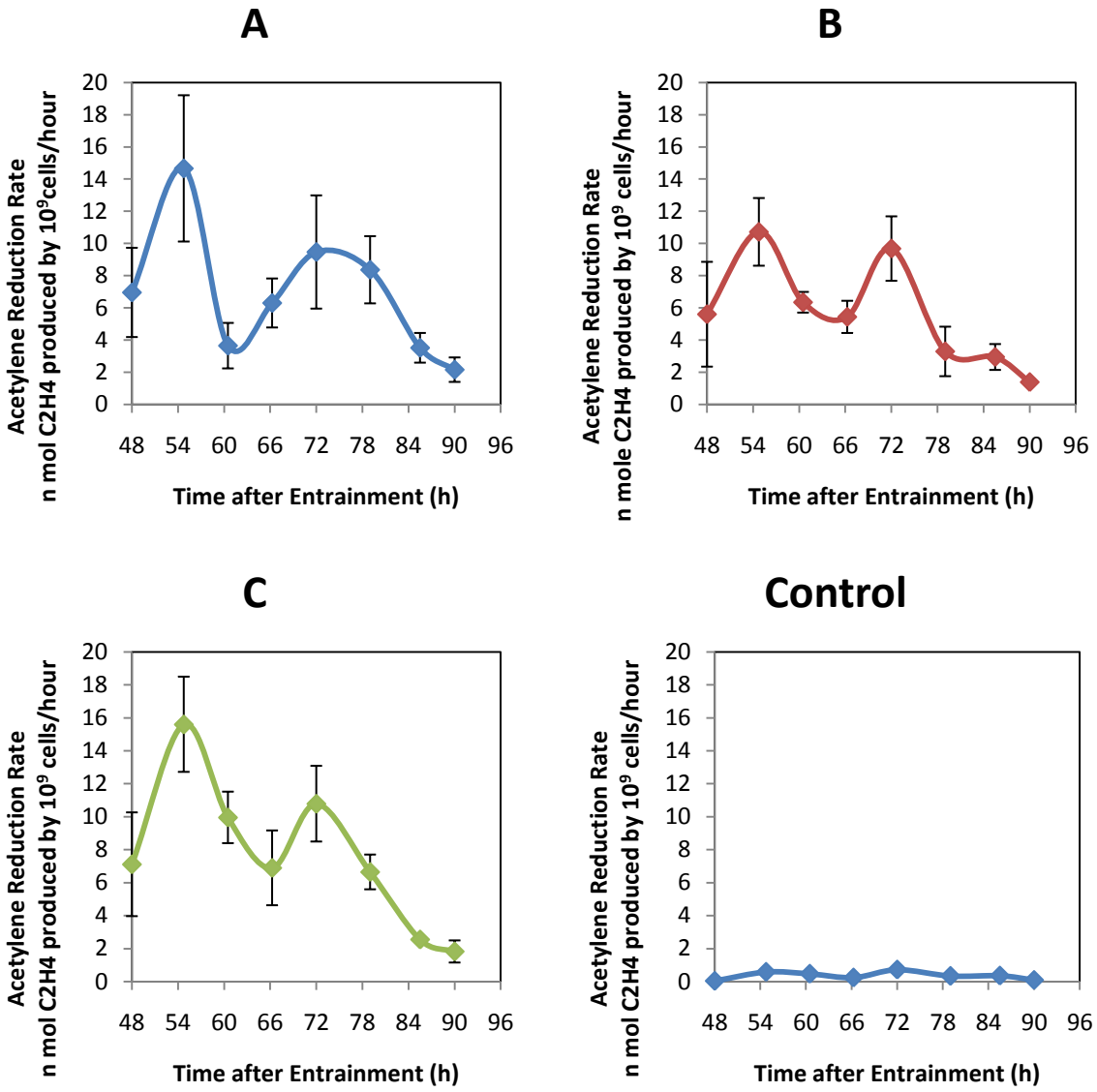

Figure 4. Nitrogenase activities after entrainments. (A) Nitrogenase activities after entrainments by light-dark cycles. (B) Nitrogenase activities after entrainments by oxygen cycles. (C) Nitrogenase activities after entrainments by light-dak and oxygen cycles.

Table 1. Doubling time of cultures at different oxygen concentrations.

O_2 Concentration	Doubling time
0	15.94h
0.01mM	16.90h
0.1mM	16.99h
1mM	20.63h