

## Full Paper

# Requirement of dark culture condition for enlargement of spheroplasts of the aerobic anoxygenic photosynthetic marine bacterium *Erythrobacter litoralis*

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In the present study, spheroplasts from the aerobic anoxygenic photosynthetic marine bacterium *Erythrobacter litoralis* were generated and cultivated. In the presence of penicillin, the spheroplasts grew and enlarged in marine broth without undergoing cell division. However, continuous light inhibited their enlargement, and they were therefore cultivated in the dark. Cellular DNA was quantified at various time points (0, 24, and 48 h) and temperatures (20°C, 25°C, and 30°C) using real-time quantitative PCR. The DNA content was highest at 30°C in the absence of penicillin, whereas there was no observable change with exposure to penicillin at all evaluated temperatures. During growth, larger spheroplasts were more frequently observed at 25°C in the presence of penicillin. These results demonstrate that the optimal culture conditions for the enlargement of spheroplasts in *E. litoralis* differ from those required for cell division.

**Key Words:** DNA replication; *Erythrobacter litoralis*; giant spheroplasts; marine bacteria; quantitative PCR

## Introduction

Appropriate growth conditions for spheroplasts from photosynthetic bacteria have not yet been reported. In this study, we generated and cultivated spheroplasts from *Erythrobacter litoralis*, an aerobic, anoxygenic, and photosynthetic marine bacterium. *Erythrobacter litoralis* is an aerobic heterotroph that belongs to the  $\alpha$ -proteobacteria (purple bacteria) group, which produces bacteriochlorophyll *a* and carotenoids (Yurkov and Beatty, 1998).

*Erythrobacter* are rod shaped and approximately  $0.4\text{--}0.5 \times 1.0\text{--}5.0 \mu\text{m}$  in size (Yurkov and Beatty, 1998).

Incubation of spheroplasts formed by lysozymes with penicillin, an inhibitor of peptidoglycan synthesis, was shown to generate giant protoplasts (Kuroda et al., 1998; Kusaka, 1967; Nakamura et al., 2011). Although DNA replication is generally related to cell division, spheroplasts have been shown to replicate their DNA without undergoing cell division under incubation conditions (Takahashi and Nishida, 2015). Furthermore, spheroplasts from *Escherichia coli* can recover to their native cellular morphology via cell wall resynthesis (Ranjit and Young, 2013).

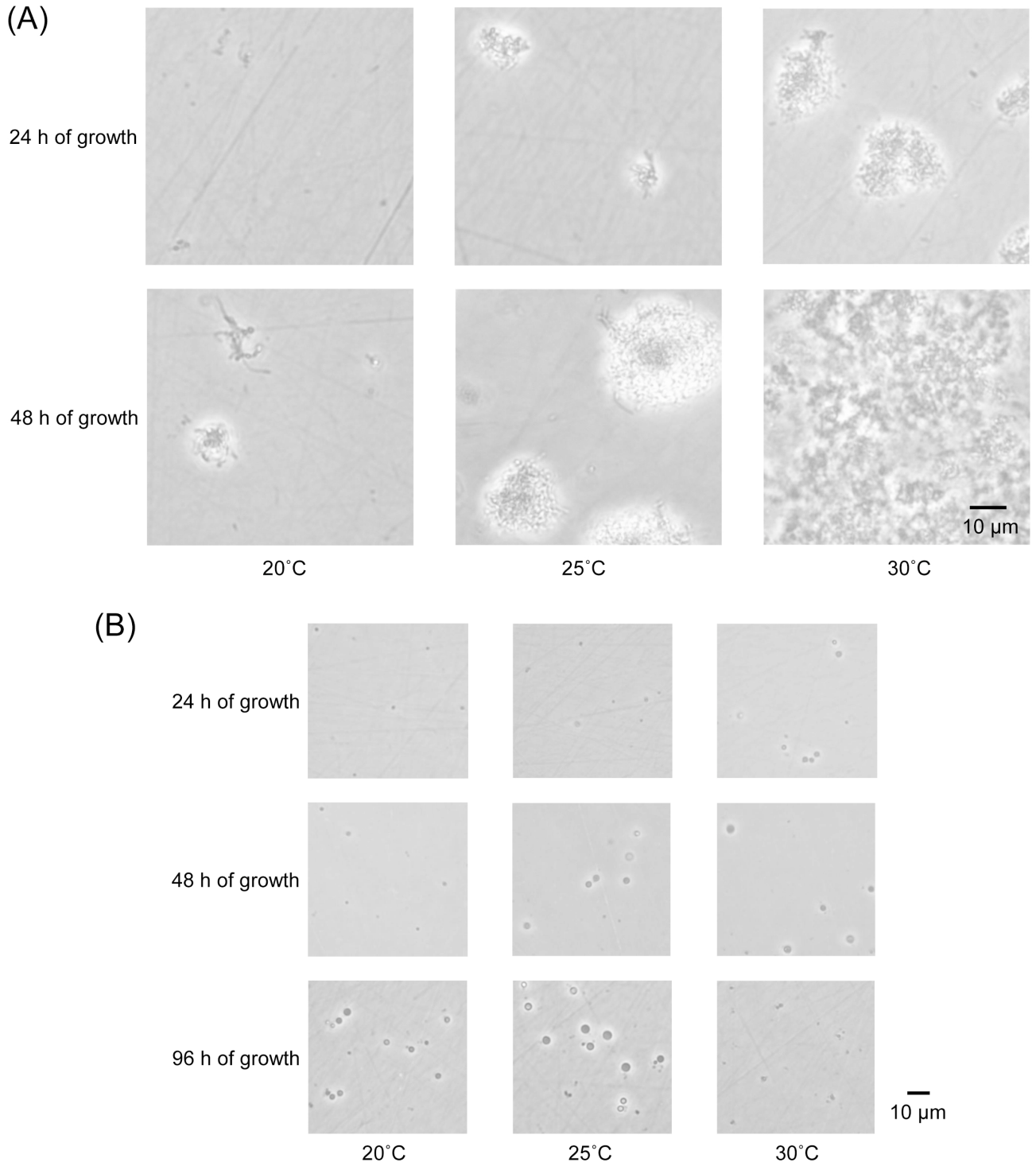
In this study, we evaluated DNA replication and cell morphology of spheroplasts from *Erythrobacter litoralis* during growth in the absence and presence of penicillin at different temperatures, in order to determine the optimal conditions for growth of *Erythrobacter litoralis* spheroplasts.

## Materials and Methods

*Cultivation of spheroplasts from Erythrobacter litoralis.* Giant spheroplasts were prepared using a previously described method with modifications (Kusaka, 1967). Spheroplast incubation was performed according to the method reported by Kuroda et al. (1998). Cells of the *Erythrobacter litoralis* NBRC 102620 strain were grown in marine broth agar (Difco Co.) under aerobic conditions. The harvested cells (0.003 g) were suspended in buffer (1 mL) consisting of 0.1 M Tris-HCl (pH 7.4) and 0.3 M sucrose. Lysozyme (Wako Co.) (200  $\mu\text{g/mL}$ ) was added to the cell suspension, which was then incubated at 25°C for 15 min. Afterwards, the suspension was divided into 2 aliquots (500  $\mu\text{L}$  each), followed by harvesting (centrifugation for 5 min at 3000 rpm) and resuspension in marine broth (500  $\mu\text{L}$ ) containing 600  $\mu\text{g/mL}$  penicillin G (Serva). The sus-

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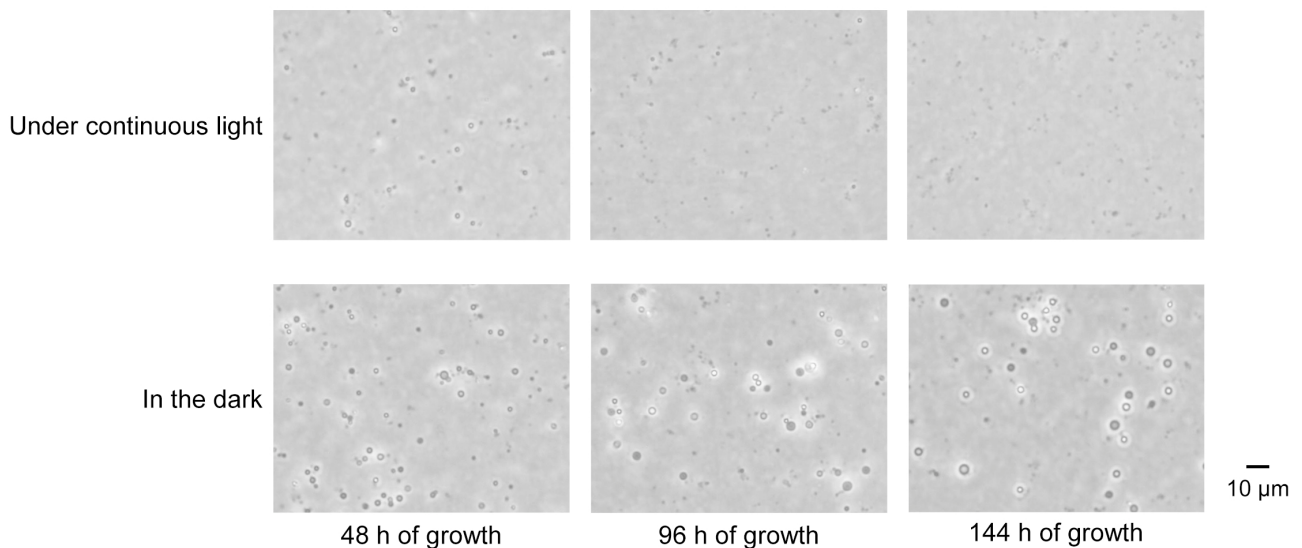


**Fig. 1.** Phase contrast micrographs of *Erythrobacter litoralis* cells.

(A) Cells in the absence of penicillin at 24 and 48 h of growth. (B) Cells in the presence of penicillin at 24, 48, and 96 h of growth. Phase contrast microscopy images were obtained using an Olympus CKX41; bar = 10 µm.

pension was then diluted by adding 2 µL of suspension to marine broth (500 µL) containing 600 µg/mL penicillin G. The diluted suspension was then incubated at 20°C, 25°C, and 30°C in the dark. Spheroplasts isolated from three different growth time points were used for further analyses: 0, 24, and 48 h. Three biological replicates were collected for each sample. The suspension was incubated at 25°C under continuous light using a fluorescent lamp. *Real-time quantitative PCR.* DNA was extracted from the

culture (500 µL) and purified using the NucleoSpin Tissue XS kit (Macherey-Nagel). DNA solution (5 µL) was used for real-time quantitative PCR. We designed primers for the DNA region near EH32\_00155 of contig 1 (5'-GCGTTTCCATCCGTTACTGT-3' and 5'-TGGT-GATGCTTGTCATTGGT-3'; PCR product size, 97 bp, primer set 1) and the DNA region near EH32\_1246 of contig 7 (5'-TTACCAGCAGCATGCACTTC-3' and 5'-GATGCAACCGGTTTCTGTTT-3'; PCR product size, 94



**Fig. 2.** Comparison of *Erythrobacter litoralis* cells cultivated in the dark with those cultivated under continuous light.

Cells were cultivated at 25°C in the presence of penicillin. Phase contrast microscopy images were obtained using an Olympus CKX41; bar = 10  $\mu$ m.

**Table 1.** Quantification cycle (Cq) values using primer set 1.

| 0 h of growth |      |      | 24 h of growth in the absence of penicillin* |      |      | 24 h of growth in the presence of penicillin |      |      | 48 h of growth in the absence of penicillin* |      |      | 48 h of growth in the presence of penicillin |      |      |
|---------------|------|------|--|------|------|--|------|------|--|------|------|--|------|------|
| 20°C          | 25°C | 30°C | 20°C   | 25°C | 30°C | 20°C   | 25°C | 30°C | 20°C   | 25°C | 30°C | 20°C   | 25°C | 30°C |
| 25.2          | 25.4 | 25.3 | 24.5   | 22.2 | 20.5 | 25.1   | 24.8 | 24.7 | 19.6   | 15.5 | 11.7 | 21.8   | 21.4 | 21.4 |
| 25.6          | 25.2 | 25.3 | 24.7   | 22.9 | 19.4 | 25.0   | 26.2 | 25.3 | 19.2   | 14.8 | 12.5 | 21.8   | 21.3 | 21.8 |
| 24.7          | 25.3 | 25.7 | 24.2   | 21.8 | 19.5 | 26.1   | 24.4 | 25.2 | 19.6   | 15.0 | 11.5 | 21.5   | 20.8 | 22.3 |

\**P* value < 0.05.

**Table 2.** Quantification cycle (Cq) values using primer set 2.

| 0 h of growth |      |      | 24 h of growth in the absence of penicillin* |      |      | 24 h of growth in the presence of penicillin |      |      | 48 h of growth in the absence of penicillin* |      |      | 48 h of growth in the presence of penicillin |      |      |
|---------------|------|------|--|------|------|--|------|------|--|------|------|--|------|------|
| 20°C          | 25°C | 30°C | 20°C   | 25°C | 30°C | 20°C   | 25°C | 30°C | 20°C   | 25°C | 30°C | 20°C   | 25°C | 30°C |
| 26.6          | 26.3 | 26.0 | 28.4   | 24.1 | 21.9 | 27.0   | 26.7 | 26.6 | 26.7   | 20.4 | 16.9 | 25.1   | 25.2 | 24.4 |
| 27.8          | 27.0 | 26.5 | 29.5   | 24.8 | 20.8 | 26.3   | 27.6 | 27.3 | 23.6   | 20.4 | 16.3 | 24.9   | 24.2 | 25.4 |
| 26.0          | 26.3 | 27.1 | 27.1   | 24.9 | 20.5 | 27.8   | 26.3 | 26.6 | 23.8   | 19.1 | 16.8 | 25.8   | 23.3 | 25.0 |

\**P* value < 0.05.

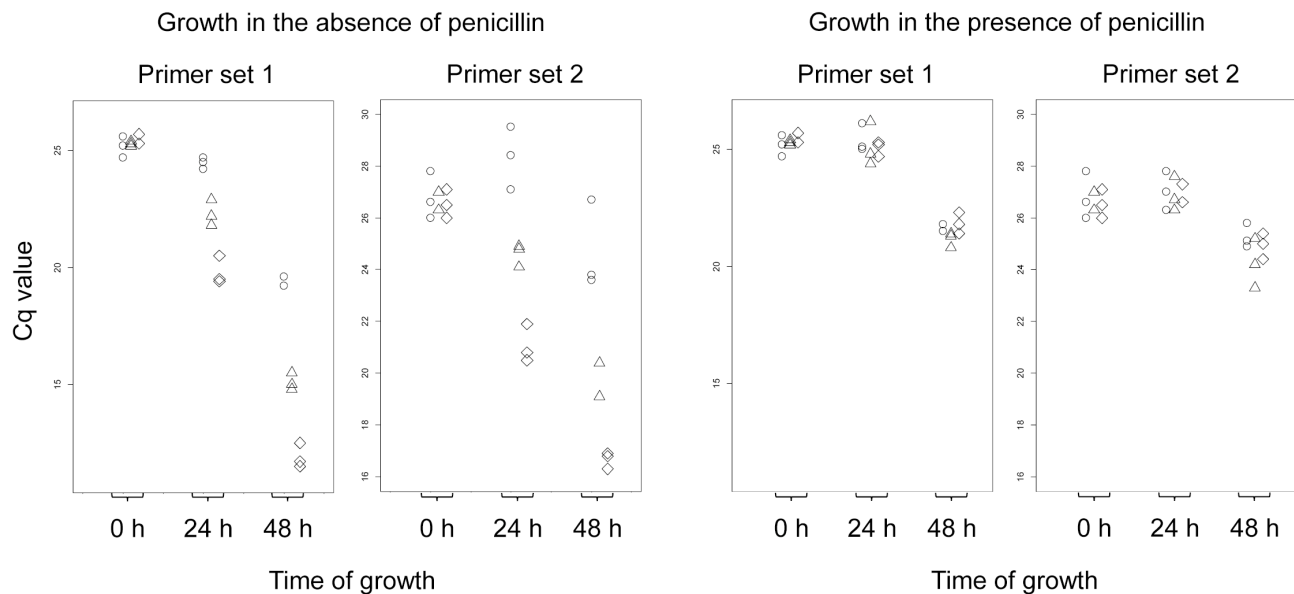
bp, primer set 2) by using the Primer3 program (<http://bioinfo.ut.ee/primer3-0.4.0/>). DNA was amplified using the FastStart Essential DNA Green Master kit (Roche) and the LightCycler Nano system (Roche). PCR was performed using the following cycling conditions: initial denaturation at 95°C for 600 s and 45 cycles of denaturation (95°C for 10 s), annealing (55°C for 10 s), and extension (72°C for 15 s). After extension, a melting curve cycle was performed from 60°C to 97°C at 0.1°C/s to confirm that non-specific products were not generated. Quantification cycle (Cq) values were obtained using the LightCycler Nano Software (Roche).

**Statistical analysis.** We performed analysis of variance (ANOVA) on Cq values from three different culture temperatures at each growth time point by using the R program (<http://www.r-project.org/>).

## Results and Discussion

Spheroplasts from *Erythrobacter litoralis* recovered their original cell morphology and divided in the absence of penicillin (Fig. 1). However, in the presence of penicillin, they did not divide, but rather enlarged (Fig. 1). Although this is consistent with previous reports of the formation of giant spheroplasts from *Bacillus subtilis* and *Escherichia coli*, the speed of enlargement was slower than observed with those bacteria (Kuroda et al., 1998; Nakamura et al., 2011). The size of the *Erythrobacter litoralis* spheroplasts was limited to a diameter of 6–7  $\mu$ m, which is smaller than that of other bacterial spheroplasts (Kuroda et al., 1998; Nakamura et al., 2011).

Light has an inhibitory effect on the synthesis of the photosynthetic apparatus of aerobic anoxygenic



**Fig. 3.** Distribution of Cq values at 0, 24, and 48 h of growth in the absence or presence of penicillin.

The circle, triangle, and rhombus symbols indicate growth at 20, 25, and 30°C, respectively. Cq values are shown in Tables 1 and 2.

phototrophs (Yurkov and Beatty, 1998). Accordingly, continuous light inhibited the enlargement of *Erythrobacter litoralis* spheroplasts (Fig. 2), suggesting that the spheroplasts maintained receptors and signal transduction pathways for light stimulus. The photosynthetic apparatus is synthesized in the dark (Harashima et al., 1987; Yurkov and Beatty, 1998) and exits within an inner (cytoplasmic) membrane (Niwa et al., 2014; Roszak et al., 2003). Therefore, it is possible that the enlarged spheroplasts generated in the dark maintained the photosynthetic apparatus. This study showed that continuous light inhibited the enlargement of *Erythrobacter* spheroplasts, suggesting that the photosynthetic apparatus may be related to the enlargement of the cell membrane.

In the absence of penicillin, 30°C was the optimal temperature for the growth of *Erythrobacter litoralis* spheroplasts (Fig. 1). However, in the presence of penicillin, 25°C was optimal for their enlargement (Fig. 1). Enlarged cells were observed after 48 h of growth at 30°C with exposure to penicillin. However, no enlargement was observed after 96 h of growth at the same temperature. This demonstrates that 30°C is not an optimal temperature for spheroplast enlargement in the presence of penicillin.

Cq values decreased during growth (Tables 1 and 2, Fig. 3), indicating an increase in the amount of DNA. The rate of increase in DNA in the absence of penicillin was higher than observed in the presence of penicillin (Fig. 3). This is consistent with our previous findings using *Escherichia coli* (Takahashi and Nishida, 2015). Therefore, the speed of DNA replication during enlargement is slower than during cell division.

The ANOVA analysis of the Cq values at various culture temperatures and time points evaluated ( $P$  value < 0.05 in 24 and 48 h of growth in the absence of penicillin and  $P$  value > 0.05 in others (Tables 1 and 2)), indicates

that DNA amounts were significantly different at 24 and 48 h in the absence of penicillin. However, they were not significantly different in the presence of penicillin. In the absence of penicillin, 30°C was also optimal for DNA replication (Fig. 2). Thus, from the viewpoint of DNA replication, the optimal growth temperature differs depending on exposure to penicillin.

Our findings demonstrate that the optimal culture conditions for enlargement of spheroplasts from *Erythrobacter litoralis* differ from those required for cell division.

#### Acknowledgments

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