Retraction

Characterization of giant spheroplasts generated from the aerobic anoxygenic photosynthetic marine bacterium *Roseobacter litoralis*

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J. Gen. Appl. Microbiol., **61**, 44–49 (2015) doi 10.2323/jgam.61.44

DNA extracted from the bacterial culture used in this paper was analyzed by the massively parallel DNA sequencing. The sequence similarity search showed that most of the obtained DNA sequences were similar to those of *Enterobacter* genomic DNA. PCR analyses using primers to detect *Enterobacter* DNA showed that the original *Roseobacter* culture was contaminated with this bacterium. The authors recognized that the cultures used in this paper were considered as a mixture of *Enterobacter* and *Roseobacter litoralis*, and the deduced results were not well rationalized. Thus, the JGAM editorial board agreed to retract the paper.

Full Paper

Characterization of giant spheroplasts generated from the aerobic anoxygenic photosynthetic marine bacterium *Roseobacter litoralis*

(Received November 18, 2014; Accepted January 9, 2015)

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We generated and characterized giant spheroplasts from the aerobic anoxygenic photosynthetic marine bacterium *Roseobacter litoralis*. The giant spheroplasts contained vacuole-like structures within the cells, mainly consisting of a single membrane. The *in vivo* absorption spectrum of the giant spheroplasts did not have peaks typically observed for bacteriochlorophyll *a*. The culture media pH decreased during the growth of the giant spheroplasts. The change in the pH profile for cells grown under light was no different from that for cells grown in the dark. These results showed that the *R. litoralis* giant spheroplasts formed lost their photosynthetic apparatus in culture. Most of the giant spheroplasts returned to their original size, likely via filamentous cells. The culture media pH increased during the growth of the filamentous cells. Some filamentous cells had septum-like structures. In such filamentous cells, DNA was separated. Initially, the color of the separated cells was white. Two weeks later, the cells changed to red in the dark, and the *in vivo* absorption spectrum of the cells had peaks typically observed for bacteriochlorophyll *a*. Our findings strongly suggest that the giant spheroplasts of *R. litoralis* can control the genetic information, return to their original cell size, and regain their original functions.

Key Words: aerobic anoxygenic photosynthesis; bacteriochlorophyll *a*; giant spheroplast; marine bacteria; *Roseobacter litoralis*

Introduction

As far as we know, giant spheroplasts of photosynthetic bacteria have not been reported. In this study, we generated and characterized giant spheroplasts from *Roseobacter litoralis*, an aerobic, anoxygenic, and photosynthetic marine bacterium. The genus *Roseobacter* (belonging to α -proteobacteria, purple bacteria) was previously proposed to comprise aerobic marine bacteria containing bacteriochlorophyll *a* and carotenoids, which contribute to the cells' red color (Shiba, 1991). The *R. litoralis* species of the genus *Roseobacter* is an aerobic heterotroph containing bacteriochlorophyll *a*, which exhibits aerobic phototrophic activity (Shiba, 1991). Cells of the species in the genus *Roseobacter* have been described as having an ovoid rod shape and a size of approximately $0.6\text{--}0.9 \times 1.2\text{--}2.0 \mu\text{m}$ (Yurkov and Beatty, 1998). The *Roseobacter* clade has also been identified as one of the major groups of marine bacteria (Buchan et al., 2005). The genome sequence of *R. litoralis* was determined in 2011 (Kalhoefer et al., 2011).

Photosynthetic purple bacteria cells are surrounded by an inner (cytoplasmic) membrane and an outer membrane. Photosynthesis-related protein complexes have been shown to exist within the inner membrane (Niwa et al., 2014; Roszak et al., 2003). The anoxygenic photosynthetic apparatus functions in the transformation of light energy into an electrochemical gradient of protons across the photosynthetic membrane (Yurkov and Beatty, 1998). It was previously suggested that the photosynthetic electron transport system might share components with the cell respiratory system (Harashima et al., 1982, 1987; Yurkov and Beatty, 1998). In addition, photosynthetic purple bacteria differ from other heterotrophic bacteria by the pres-

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None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Fig. 1. Micrographs (A) and fluorescence micrographs (B) of the spheroplasts, the giant spheroplasts, and the filamentous cells.

Lysozyme (200 $\mu\text{g}/\text{mL}$) was added to the cell suspension, and the suspension was shaken at 29°C for 15 min at 60 rpm. The cells were harvested and resuspended in a medium consisting of marine broth and 0.2 M NaCl. A 1/57 volume of this suspension was diluted into a medium consisting of marine broth, 0.2 M NaCl, and 600 $\mu\text{g}/\text{mL}$ of penicillin G. The suspension was shaken at 26°C at 30 rpm. The giant spheroplasts and filamentous cells were collected at 24 h and 56 h, respectively. Fluorescence image of the cells stained with DAPI. DAPI (final concentration, 0.17 $\mu\text{g}/\text{mL}$) was added to a suspension of the cells and incubated at room temperature for 3 min. Fluorescent micrographs were taken using a laser scanning microscope (LSM510 META, Zesis, Germany) with plane scan mode (Objective: C-Apochromat 40 $\times$ /1.2 W corr; Filters: BP 420–480; IR, BP 505–530; Beam Splitter: HFT 405/488; Blue diode laser: 405 nm 56.5%; Argon laser: 488 nm 5.0%).

ence of bacteriochlorophyll *a*, as indicated by absorption peaks, which range from 800 to 880 nm in cell suspensions (Yurkov and Beatty, 1998). The absorption spectra of *Roseobacter* cells were previously shown to yield two peaks at 806 and 868 nm (Yurkov and Beatty, 1998).

Bacterial L-forms and protoplasts (or spheroplasts) lack a cell wall. Although protoplasts do not normally undergo

prolonged growth or division, L-form cells divide via various processes, including membrane blebbing, tabulation, vesiculation, and fission (Errington, 2013). Although the cell wall is a crucial structure in most of bacteria, it is a wonder why the cell wall lacking bacteria can live. Recovery of spheroplasts of *Escherichia coli* has been reported (Ranjit and Young, 2013). In addition, recovery of

Fig. 2. Electron micrographs of the giant spheroplasts (A) and a filamentous cell from the giant spheroplasts (B).

The giant spheroplasts were used at 24 h of growth. IM, OM, and V indicate the inner membrane, outer membrane, and vacuole-like structure, respectively. Partial formation of the cell wall and outer membrane, and the transfer of nucleoid were observed at the early stage of a filamentous cell from a giant spheroplast.

L-forms has also been reported (Billings et al., 2014; Kawai et al., 2014). Incubation of protoplasts formed by lysozymes in the presence of penicillin (an inhibitor of peptidoglycan synthesis) was shown to generate giant protoplasts (Kuroda et al., 1998; Kusaka, 1967; Nakamura et al., 2011). However, protoplasts do not divide under incubation, but DNA replication has been shown to occur. Thus, many copies of DNA exist in giant protoplast cells (Nakamura et al., 2011). In this study, we investigate whether the giant spheroplasts can return to the original cells.

Materials and Methods

Preparation of giant spheroplasts from R. litoralis. Giant spheroplasts were prepared using a modified version of the method previously described by Kusaka (1967) and the spheroplast incubation method reported by Kuroda et al. (1998). Cells of *R. litoralis* NBRC 15278 (type strain) were grown in a marine broth agar (Difco Co.) under aerobic conditions. The harvested cells (0.003 g) were suspended in a buffer (1 mL) consisting of 0.1 M Tris-HCl (pH 7.4) and 0.3 M sucrose. Lysozyme (Wako Co.) (200

Fig. 3. Absorption spectra of the normal *Roseobacter littoralis* cells, the spheroplasts at 0, 6, 18, 190, and 400 h of growth under light and in the dark.

The *in vivo* absorption was measured using a Shimadzu UV-2450 spectrophotometer, scanning between 400 nm and 1000 nm.

$\mu\text{g/mL}$) was added to the cell suspension, and the suspension was shaken at 29°C for 15 min at 60 rpm. The cells were then harvested (centrifugation for 5 min at 3000 rpm) and resuspended in a medium consisting of marine broth (Difco Co.) and 0.2 M NaCl. A 1/57 volume of this suspension was diluted into a medium consisting of marine broth, 0.2 M NaCl, and 600 $\mu\text{g/mL}$ penicillin G (Serva Co.). The suspension was shaken at 26°C at 30 rpm under light (using a fluorescent lamp) and in the dark.

Microscopy. Light microscope observations were made using an OLYMPUS CKX41 (Japan). The giant spheroplasts and the filamentous cells were prepared from *R. littoralis* cells for 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. DAPI (final concentration, 0.17 $\mu\text{g/mL}$) was added to the suspension of giant spheroplasts and filamentous cells and incubated at room temperature for 3 min. Fluorescent micrographs were taken using a laser scanning microscope (LSM510 META, Zeiss, Germany) with plane scan mode (Objective: C-Apochromat 40 $\times$ /1.2 W corr; Filters: BP 420–480 IR, BP 505–530; Beam Splitter: HFT 405/488; Blue diode laser: 405 nm 56.5%; Argon laser: 488 nm 5.0%).

Transmission electron microscopy. Giant spheroplasts were pre-fixed by adding 2% glutaraldehyde in 0.1 M phosphate buffer. The cells were then post-fixed with 2% OsO_4 in 0.1 M phosphate buffer for 2 h at 4°C. After fixation, the cells were washed three times with 0.1 M phosphate buffer. Next, the cells were dehydrated with ethanol, followed by dehydration with propylene oxide and embedding in Epon812 (Shell Chemical). The cells were stained with uranyl acetate for 15 min and then with lead for 5 min. They were examined with a Hitachi H-7600 transmission electron microscope at 100 kV.

***In vivo* absorption and media pH measurements.** The *in vivo* absorption (Yurkov and Beatty, 1998) was measured using a Shimadzu UV-2450 spectrophotometer, with scanning between 400 nm and 1000 nm. The pH of the culture media was measured using a Horiba Laqua pH meter. The pH measurements were taken for 12 samples of

spheroplasts under light and 12 samples in the dark. In addition, the culture media of 9 samples of normal cells under light and 9 samples in the dark were measured as controls.

Results and Discussion

Incubation of spheroplasts formed by lysozymes in the presence of penicillin was shown to grow them and generate giant spheroplasts between 2 and 24 h of growth (Fig. 1A). In this study, the size of the *R. littoralis* giant spheroplasts produced was limited to approximately 15 μm in diameter (Fig. 1A). Light microscope observation showed that cell size did not change after 14–18 h of growth. In previous studies, it was shown that the giant spheroplasts (protoplasts) of the Gram-positive bacterium, *Bacillus subtilis*, and the Gram-negative bacterium, *Escherichia coli*, contain vacuole-like structures (Kuroda et al., 1998; Nakamura et al., 2011). Here, we found that the giant spheroplasts of *R. littoralis* also contained some vacuole-like structures (Figs. 1A and 2A). To our knowledge, this is the first reported study to generate giant spheroplasts from photosynthetic bacteria.

Evaluation with transmission electron microscopy showed that the outer membrane of the giant spheroplast was partially removed (Fig. 2A). In *E. coli*, the outer membrane proteins play an important role in the cell wall synthesis (Paradis-Bleau et al., 2010; Typas et al., 2010). Considering that the photosynthetic apparatus is located in the inner membrane, this observation suggests that if the photosynthetic apparatus is functional within the giant spheroplasts, then protons are transported outside the cell during the photosynthetic process.

Light has an inhibitory effect on the photosynthetic apparatus synthesis in aerobic anoxygenic phototrophs (Yurkov and Beatty, 1998). Thus, we used the normal *Roseobacter littoralis* cells, the spheroplasts at 0, 6, 18, 190, and 400 h of growth under light and in the dark to detect bacteriochlorophyll *a*. Measurement of the *in vivo*

Fig. 4. Change in the pH of the culture media from giant spheroplasts grown under light and in the dark.

Samples prepared using two culture conditions, growth under light and growth in the dark, were evaluated for changes in pH. The pH measurements were taken for 12 samples of spheroplasts under light and 12 samples in the dark at 0, 2, 6, 21, 24, 50, 56, 70, 78, and 104 h of growth. In addition, the culture media of 9 samples of normal cells under light and 9 samples in the dark were measured as a control at 0, 6, 12, and 18 h of growth.

absorption spectra of the normal *R. litoralis* cells and the spheroplasts at 0 h of growth showed peaks comparable to those previously reported for bacteriochlorophyll *a* (at 807 nm and 878 nm) (Shiba, 1991; Tang et al., 2009); however, no peaks were observed for the giant spheroplasts (Fig. 3). This observation strongly suggested that the giant spheroplasts lost their photosynthetic apparatus.

The medium pH profiles for cultures of normal cells and spheroplasts differed (Fig. 4). There was no difference observed in the medium pH profile for cultures of normal cells grown under light and those grown in the dark (Fig. 4). It is suggested that protons are held between the inner and outer membranes in *R. litoralis* in the photosynthetic and respiratory systems. Thus, in normal *R. litoralis* cells, all transported protons cannot be detected by monitoring the medium pH. However, in the spheroplasts, all transported protons can be detected because of the lack of an outer membrane and cell wall.

The pH of the spheroplast media decreased between 0 h and 50 h of growth (Fig. 4). This decrease in the pH was likely caused by protons transported outside the cell in the photosynthetic and/or respiratory systems. If the photosynthetic apparatus was functional, the media pH would decrease more when grown under light than in the dark. Although the medium pH profile for cultures grown in the dark was more varied than that under light, those pro-

files were very similar (Fig. 4). This result was consistent with the lack of bacteriochlorophyll *a* in the inner membrane (Fig. 3). The giant spheroplasts of *R. litoralis* probably lost their ability for photosynthesis.

Photosynthesis in *R. litoralis* occurs in a manner similar to that of respiration. Thus, in the photosynthesis and respiratory systems, protons are transported outside the cell, and a proton concentration gradient is formed across the cytoplasmic membrane. It was previously suggested that the photosynthetic electron transport system might share components with the respiratory system (Harashima et al., 1982, 1987; Yurkov and Beatty, 1998). However, our findings suggest that the giant spheroplasts of *R. litoralis* tended to select the respiratory system and remove the photosynthetic system.

R. litoralis is an aerobic heterotroph. Thus, the respiratory system is essential for energy production in this bacterium. Our findings confirmed that the photosynthetic system serves as a subsystem for energy production. *R. litoralis* may be a key organism for elucidating the evolutionary relationship between photosynthesis and the respiratory system.

L-form cells divide via various processes, including membrane blebbing, tabulation, vesiculation, and fission (Errington, 2013). In this study, however, microscopic observation showed that most of the giant spheroplasts

Fig. 5. Recovery from the giant spheroplasts of *Roseobacter littoralis*: a hypothetical model.

changed to filamentous cells (Figs. 1A, 1B, and 2B). Although elongated cells are also found in the recovery of (small) spheroplasts of *E. coli* (Ranjit and Young, 2013), the length is much shorter than that of the filamentous cells of *R. littoralis*. The culture media pH increased between 56 h and 78 h of growth (Fig. 4). In this period, filamentous cells appeared (Fig. 1A). It strongly suggested that a large amount of ATP was synthesized for growth of the filamentous cells. After filamentation, some cells generated septum-like structures (Fig. 1B). The fluorescence image showed that giant spheroplasts contained DNAs; however, vacuole-like structures were not stained with DAPI (Fig. 1B). In some filamentous cells, the DNAs were separated (Fig. 1B). Transmission electron microscope observation showed that the filamentation was accompanied by cell wall synthesis and nucleoid transfer (Fig. 2B).

Initially, the color of the cells separated from the filamentous cells was white. In addition, the *in vivo* absorption spectrum of the white cells had no peaks typically observed for bacteriochlorophyll *a* (Fig. 3). At 400 h of growth, the cells changed to red (carotenoid synthesis) in the dark, and the *in vivo* absorption spectrum of the cells had peaks typically observed for bacteriochlorophyll *a* (Fig. 3). On the other hand, the cells did not change under light (Fig. 3). This indicates that the cells separated from the filamentous cells are able to synthesize bacteriochlorophyll *a*. However, the continuous light inhibits the synthesis.

Our findings strongly suggest that the giant spheroplasts of *R. littoralis* can control the genetic information, return to their original cell size, and regain their original functions (Fig. 5). Normally, *R. littoralis* grows by a symmetry binary fission. On the other hand, during recovery from the giant spheroplasts of *R. littoralis*, the cells were elongated probably because of the genetic information distribution. This system may be a prototype of cell division.

Acknowledgments

This work was supported by a grant from The Cannon Foundation.

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