Pyocyanine as a Potent Inhibitor for the Growth of *Rhodopseudomonas sphaeroides* B5

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**Abstract.** Pyocyanine severely inhibited the growth of *Rhodopseudomonas sphaeroides* B5 under photosynthetic conditions. Under these conditions, pyocyanine at concentrations of 0.05 and 0.1 μg/ml gave rise to about 61% and 99% inhibition, respectively, whereas pyocyanine at a concentration of 1 μg/ml was required to inhibit the growth by about 55% under aerobic dark conditions. Antibiotic action of pyocyanine under photosynthetic conditions was reversible and not bactericidal. Degradation of carotenoid was observed in pyocyanine-treated cultures.

It is well known that pyocyanine, one of the phenazine derivatives, is produced by *Pseudomonas aeruginosa*. Pyocyanine has antibiotic activities against bacteria [1, 2, 4] and animals [2]. It was also reported that the direct reduction of pyocyanine by NADH diverted electron flow and increased the production of O₂⁻ and H₂O₂, and that the antibiotic action of this pigment was largely a reflection of the toxicity of these products of oxygen reduction [4]. Baron and Rowe [1] reported that pyocyanine was bactericidal for all susceptible organisms and that facultative anaerobes were more resistant to the action of pyocyanine under fermentative conditions than under aerobic conditions.

During the course of the isolation of photosynthetic bacteria, we found that pyocyanine produced by *Pseudomonas* severely inhibited growth of photosynthetic bacteria under photosynthetic conditions. In this report, we present an antibiotic action of pyocyanine to a photosynthetic bacterium, *Rhodopseudomonas sphaeroides* B5, under these conditions. This bacterium may be classified as *Rhodobacter* [6], but in this report we use the name of *Rhodopseudomonas*, because photosynthetic bacteria are not classified in Volumes 1 [9] and 2 [13] of Bergey's Manual of Systematic Bacteriology (9th edition); these volumes were already published.

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**Materials and Methods**

**Bacterial strain, medium, and growth conditions.** *Rhodopseudomonas sphaeroides* B5, which was isolated by Watanabe et al. [14], was used throughout the experiments.

For the culture of the bacterium, the minimal medium of Ormerod et al. [11] was used with the following modifications. Thiamine-HCl (1 mg/liter) and nicotinic acid (1 mg/liter) were added to the medium, and the amount of DL-malic acid was changed to 4.02 g/liter.

For testing the effect of pyocyanine on the growth of the bacterium, cells grown in the synthetic medium were inoculated into test tubes containing the synthetic medium and various concentrations of pyocyanine, if necessary. Aerobic growth conditions were performed by shaking in the dark. For anaerobic cultivation, test tubes were closed with a butyl-rubber stopper, and the atmosphere inside the tubes was changed to argon. Test tubes were then placed in a water bath, which was illuminated (about 10 klux intensity) by incandescent bulbs. All of the incubations of cells were carried out at 30°C. Bacterial growth was followed by optical density at 660 nm with a Hiranuma photoelectric colorimeter EPO-B (Hiranuma Sangyo Co., Ltd., Mito, Japan).

**Preparation of pyocyanine.** Pyocyanine was prepared from phenazine methosulfate by a photooxidation procedure [8] and purified by the method of Jagendorf and Margulies [7].

**Measurement of absorption spectra of pigments.** Cells anaerobically incubated in the presence or absence of pyocyanine under light and anaerobic conditions were harvested, and pigments were extracted twice with acetone–methanol (7:2, vol/vol), as described by Clayton [3]. Absorption spectra of extracts were monitored by a Hitachi spectrophotometer 124 (Hitachi Ltd., Tokyo, Japan).
Fig. 1. Effect of pyocyanine on growth of *Rhodopseudomonas sphaeroides* B5 under anaerobic and aerobic conditions. Cells were cultivated in the absence or presence of pyocyanine as described in the text. (A) Growth under light and anaerobic conditions. Concentration of pyocyanine (µg/ml): 0 (absence), 0; 0.02, ◯; 0.03, ◼; 0.05, △; 0.1, □. (B) Growth under dark and aerobic conditions. Concentration of pyocyanine (µg/ml): 0 (absence), 0; 0.2, ◯; 0.5, ◼; 1.0, △; 2.0, □.

Results

It was observed that pyocyanine intensively inhibited growth of the photosynthetic bacterium under anaerobic conditions. Under these conditions, pyocyanine at concentrations of 0.05 and 0.1 µg/ml gave rise to 61% and 99% inhibition after 10.5 h of incubation, respectively (Fig. 1A). On the other hand, under aerobic conditions, pyocyanine at a concentration of 0.1 µg/ml inhibited growth only slightly, and at a concentration of 1 µg/ml 55% inhibition was observed after 11 h of incubation (Fig. 1B). It was also observed that 2 h of illumination of an anaerobic culture of the bacterium in the presence of pyocyanine (0.1 µg/ml) brought about a remarkable color change of the culture from brown to pale yellow (data not shown). Therefore, absorption spectra of pigments from cells that were anaerobically incubated in the presence or absence of pyocyanine were examined.

As shown in Fig. 2A and B, absorption spectra of pigments extracted from pyocyanine-treated cells, especially at about 487 and 457 nm, were obviously different from those of cells incubated without pyocyanine. Alteration in absorption spectra in the carotenoid region (from about 450 to 490 nm) were dependent on the concentration of pyocyanine and duration of pyocyanine treatment. These results indicate that illumination in the presence of pyocyanine gave rise to decomposition of carotenoid(s). No change of absorption spectrum was observed in the carotenoid region when an acetone-methanol extract of cells was illuminated in the presence of pyocyanine (0.5 µg/ml) in the dark, in the presence of pyocyanine (0.5 µg/ml) in the light, or in the absence of pyocyanine in the light.

Viability of a culture that was incubated for 2 h in the presence of pyocyanine at a concentration of 0.5 µg/ml under light and anaerobic conditions was essentially identical with that of the culture before the incubation (data not shown). This indicated that the antibiotic action of pyocyanine, in this case, was reversible and not bactericidal.

Fig. 2. Absorption spectra of acetone-methanol extracts from *Rhodopseudomonas sphaeroides* B5. Bar represents absorbance of 0.02. (A) Cells were incubated under light and anaerobic conditions for 15 min in the absence or presence of various concentrations of pyocyanine. (B) Cells were incubated under light and anaerobic conditions in the presence of pyocyanine (0.1 µg/ml) for certain periods. Pigments were extracted from these cells, and absorption spectra were monitored (A and B). (C) Pigments were extracted from cells cultivated in the absence of pyocyanine. Absorption spectra of extracts were monitored after extracts were anaerobically incubated for 30 min in the presence of pyocyanine (0.5 µg/ml) in the dark, in the presence of pyocyanine (0.5 µg/ml) in the light, or in the absence of pyocyanine in the light.