Isolation and identification of antialgal substances produced by
*Pseudomonas aeruginosa*

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**Abstract**

*Pseudomonas aeruginosa* strongly inhibited the growth of green microalgae and cyanobacteria by the release of low molecular weight, thermoresistant factors. The antialgal substances were resistant to various enzymes and remained active in agar after a 3-months storage period at 4 °C in the absence of light. The results indicate that inhibition of algal growth was mediated by the phenazine pigments released by the bacterium. Pyocyanine and an unidentified pale blue pigment had no effect on algal growth, whereas 1-hydroxyphenazine and oxychlororaphine showed strong antialgal activity.

**Introduction**

Various situations in which bacteria exhibit antagonistic effects against microalgae have been described (Jones, 1982; Colwell & Speidel, 1985; Hayashida et al., 1991). However, only in a few cases has the nature of the antagonistic mechanisms been elucidated (Berger et al., 1979; Saks & Kahn, 1979; Watson et al., 1986). Our recent observations indicated that *Pseudomonas aeruginosa* strongly inhibits the growth of various green microalgae and cyanobacteria by the release of agar-diffusible pigmented products (Dakhama et al., 1989). Although similar observations have been reported elsewhere (Stingfellow & Pratt, 1988), no information was given on the nature of the inhibitory agent. Indeed, *P. aeruginosa* is known to produce a number of antibiotic substances such as diverse phenazine pigments (Sierra & Veringa, 1958; Chang & Blackwood, 1969; Gerber, 1969; Herbert & Holliman, 1969; Holliman, 1969; Watson et al., 1986), pyo compounds (Hays et al., 1945) and glycolipids (Hisatsuka et al., 1971). However, all these substances were detected as a result of their bactericidal activity while antialgal activity was not tested.

The present investigation was undertaken to characterize the previously observed *P. aeruginosa*-mediated microalgal growth inhibition, and to determine the nature of the antialgal substances produced by this bacterium.

**Materials and methods**

**Organisms and growth conditions**

The microalgae, *Chlorella* sp., *Chlorella vulgaris*, *Klebsormidium flaccidum*, *Scenedesmus obliquus*, and the cyanobacteria, *Anabaena* sp. and *Phor-
**midium bohneri**, were isolated in our laboratory. *Ankistrodesmus falcatus* (S/ANKIS-1, SERI microalgal culture collection, Colorado, USA), *Oscillatoria agardhii* (UWCC FW 277, Department of Botany, University of Washington, Seattle, Wash., USA) and *Scenedesmus bicuspidalis* (initially named *S. ecoris* UTEX LB 1359) were obtained from culture collections. *S. bicuspidalis* was then rendered axenic by serial dilution.

Algal cultures were grown at 25 °C under cool-white fluorescent lamps (200 μmol photon m\(^{-2}\) s\(^{-1}\), 10:14 LD cycle), in a mineral medium (Dauta, 1982) used either as a liquid or as a solid medium after the addition of 1.5% (w/v) of Bacto-agar (Difco Laboratories Inc., Detroit, MI).

**Pseudomonas aeruginosa** was isolated on nutrient agar, from a culture of *Anabaena sp.*, and was identified with API rapid NFT (Analytab Products, Plainview, N.Y.). The following culture media (all purchased from Difco) were used for bacterial growth: nutrient agar (NA), nutrient broth (NB), Pseudomonas isolation agar (PIA), Pseudomonas agar F (PAF) and Pseudomonas agar P (PAP). Unless otherwise stated, the growth temperature was 25 °C for NA and 30 °C for PIA cultures.

**Algal growth response to P. aeruginosa**

The effects of *P. aeruginosa* on the growth of algae were detected on solid media, using two methods: the agar-disc and the agar-drop methods. In the agar-disc method, agar-containing mineral medium was poured into Petri dishes and allowed to solidify. Algal inoculum (8 ml of melted algal agar inoculated with 2 ml of one-week algal culture) was poured over hardened base in the Petri dish and allowed to solidify at room temperature. Inocula of the bacterial strains were standardized as follows: bacteria were subcultured from nutrient agar stock cultures into nutrient broth and incubated at 25 °C until the suspension reached an optical density of 0.65 at 520 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. Then, tubes containing 8 ml of nutrient agar were inoculated with 2 ml of the standardized bacterial suspension, mixed vigorously and poured over the hardened base of nutrient agar in Petri dish. After a growth period of 48 h, discs of agar were cut out from these plates with the large end of a sterile Pasteur pipet and transferred on algae-seeded agar plates.

The agar-drop method was used to quantify the degree of the algal response to an increasing bacterial inoculum. This method consisted of a serial (decimal) dilution of the standardized bacterial suspension in sterile nutrient agar. Aliquots (100 μl) of each dilution were dropped in the bottom of Petri dishes, allowed to solidify and covered with solid algal medium. After 48 h of bacterial growth, the plates were seeded with algal inoculum as described above. Observations of algal growth were made periodically over a period of two weeks. Zones of enhancement or inhibition surrounding each disc or drop were noted and compared with controls consisting of discs or drops of sterile nutrient agar.

**Characterization of the inhibitors**

**Molecular weight estimation**

The molecular weight of the inhibitors was estimated by their ability to diffuse through Spectrapor dialysis tubing (Spectrum Medical Industries, Los Angeles, CA) of differing molecular weight cut-offs (1000, 3500, 10000). The membranes were cut to fit Petri dishes, sterilized by autoclaving and inserted between two layers of nutrient agar. *P. aeruginosa* was grown for 48 h on the surface of the top layer, avoiding direct contact with the membrane, and inhibition tests were performed using agar discs taken from the bottom layer containing only substances which diffused through the agar and the membrane. The discs were placed on algae-seeded plates incubated at 25 °C under controlled illumination (200 μmol photon m\(^{-2}\) s\(^{-1}\), 10:14 L:D cycle). The plates were monitored daily for inhibitory activity.

All subsequent tests were carried out using the bottom layer of agar containing bacterial prod-