Light-mediated Changes in Pigmentation of *Pseudomonas aeruginosa* Cultures

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(Received 25 January 1979)

Cultures of *Pseudomonas aeruginosa* PAO grown under uninterrupted broad-spectrum light showed different pigmentation from dark-grown cultures. Whereas dark-grown bacteria produced pigments which resulted in blue–purple coloured agar, light-grown organisms produced red coloured plates. Extraction and quantification of pigments showed that both dark- and light-grown cultures produced similar concentrations of pyorubrin (red) and pyoverdin (yellow). In contrast, the concentration of pyocyanin (blue) was substantially reduced under certain lighting conditions. This decrease was dependent on both the light intensity and wavelength and occurred with light in the ultraviolet and violet region of the spectrum. After its release from bacteria, pyocyanin was rapidly and non-reversibly photoinactivated with first-order kinetics to produce colourless photoproduct(s).

INTRODUCTION

While studying the general effect of visible and near-visible light on bacteria, we observed that cultures of *Pseudomonas aeruginosa* produced different coloured agar plates when grown under light and dark conditions. We presumed that this reflected changes in the diffusible pigments present. Since light-mediated effects in micro-organisms are of interest (see Zelle & Hollaender, 1955; Leach, 1971; Jagger, 1972; Taylor & Koshland, 1975; Thomas, 1977) and since pigment production is a characteristic commonly used for identifying and differentiating pseudomonads, the following study was undertaken.

METHODS

Cultures. *Pseudomonas aeruginosa* strain PAO, obtained from P. V. Phibbs (Virginia Commonwealth University), was used for all studies presented in this paper. Major findings were confirmed using *P. aeruginosa* strain SC8822 obtained from R. J. McRipley (E. R. Squibb).

Growth conditions. Medium A (King *et al.*, 1954) was used in this study. This medium was supplemented with 1 mM-CuCl to enhance pigment production (King *et al.*, 1954). Similar results were obtained with Pseudosel agar (BBL), except that production of pyorubrin was depressed. Disposable plastic Petri dishes (approx. 10 × 50 mm; Millipore) containing 4 ml medium were surface inoculated with about 2×10^7 bacteria. Cultures were grown at 25 °C for specified periods in the presence or absence of light (see below). The plastic Petri dishes transmitted 90% of the incident light above 360 nm.

Assays. Pigments were extracted and assayed as previously described by Palumbo (1972). Basically, the technique involved chloroform extraction of the medium to separate pyocyanin, followed by aqueous extraction of the medium to remove pyoverdin and pyorubrin together. For spectrophotometric determination, pyocyanin was extracted from chloroform into 0.2 M-aqueous HCl, and absorbance at 660 nm was determined after neutralization to pH 7.0. Pyorubrin was measured in aqueous extracts by absorbance at 525 nm. Pyoverdin was measured in aqueous extracts by fluorescence at 525 nm after excitation at 410 nm (pH 8.0 to 8.2).

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Fig. 1. Effect of light wavelength and intensity on pyocyanin production. Bacteria were inoculated on to plates and grown under light of different wavelengths (○, 371 nm; □, 420 nm; ×, 550 nm; ■, 660 nm; ●, 740 nm) at the specified intensities for 5 d. Results are expressed as the ratio of pigment produced under lighted conditions to pigment produced by dark-grown controls. Representative ranges ($\pm$ one standard error) for each point have been calculated for 371 nm light. Results are averages of three experiments.

Lighting. One broad-spectrum (350 to 700 nm) and five narrow-bandwidth (371, 420, 550, 660 and 740 nm) fluorescent lights were used. Light intensity was controlled by adjusting the distance between the lights and the irradiated object. Irradiance was measured at the level of the object using a Tektronix J16 Photometer/Radiometer with a J6512 probe. The spectral characteristics of these lights and the techniques for measurement of light intensity have previously been described in detail (Propst-Ricciuti & Kenny, 1974; Siebert *et al.*, 1975).

RESULTS

*General response of *P. aeruginosa* to broad-spectrum light*

Pseudomonas aeruginosa PAO grown on agar medium in the dark normally produces three characteristic diffusible pigments: (1) pyocyanin, a non-fluorescent blue pigment; (2) pyorubrin, a non-fluorescent red pigment; (3) pyoverdin (fluorescein), a fluorescent yellow pigment. Studies of the effects of broad-spectrum light ($2400 \mu\text{W cm}^{-2}$) on *P. aeruginosa* grown for 4 d at room temperature showed dramatic changes in pigmentation. Whereas dark-grown organisms produced diffusible pigments which resulted in blue-purple coloured agar, light-grown organisms produced red coloured plates. The pigments produced were extracted and quantified. The ratios ($\pm$ one standard error) of the amounts of pyocyanin, pyorubrin and pyoverdin produced by light-grown organisms to the amounts produced by dark-grown organisms were, respectively, 0.52 ± 0.02 , 0.99 ± 0.02 , and 0.94 ± 0.05 . Thus only pyocyanin was affected by broad-spectrum light under the conditions tested.

Effect of light wavelength and intensity on pigmentation

To determine the effects of the wavelength and intensity of light on the decrease in pyocyanin concentration and to investigate more fully the possible effect of light on pyorubrin and pyoverdin, cultures of *P. aeruginosa* were grown under different intensities of light at 371, 420, 550, 660 and 740 nm for 5 d and then pigments were extracted and quantified. In support of the results obtained with broad-spectrum light, pyorubrin and pyoverdin were either unaffected or only marginally influenced by light intensity and wavelength when studied from 0 to $600 \mu\text{W cm}^{-2}$ with the above narrow-bandwidth fluorescent lights (data not presented). The concentration of pyocyanin, however, was substantially reduced under

Table 1. *Effect of treatment conditions on pyocyanin concentration*

The medium used for plates was not exposed to light after preparation, except as indicated. Pre-treated plates were irradiated for 3 d just prior to inoculation. After inoculation, all plates were incubated for 3 d under light or dark conditions, as appropriate. Bacteria were removed from the agar surface of all plates by flushing with distilled water prior to extraction procedures. For post-treated plates, bacteria were removed from the agar surface by first flushing with sterile distilled water, then with 95 % ethanol to prevent regrowth and finally with more sterile distilled water (control studies had shown that this procedure did not affect the coloration of agar plates); the plates were then exposed to light for an additional 2 d. Broad-spectrum light at $1500 \mu\text{W cm}^{-2}$ was used throughout. Results are averages of three experiments $\pm$ one standard error.

Medium treatment/growth conditions				Pyocyanin (A_{660} units)
Pre-treated	Growth	Post-treated	Plate colour	
Dark	Dark	NA	Blue	0.25 (± 0.01)
Dark	Light	NA	Red	0.17 (± 0.01)
Light	Dark	NA	Blue	0.30 (± 0.02)
Dark	Dark	Light	Red	0.08 (± 0.02)

NA, Not applicable.

certain conditions of lighting (Fig. 1). The decrease in pyocyanin was dependent both on the intensity and on the wavelength of the light. Light with wavelengths in the ultraviolet and violet regions of the spectrum was primarily responsible for the decrease in pyocyanin concentration.

Nature of light-mediated decrease in pyocyanin

The effect of light on pyocyanin concentration could be due to a number of interactions including an indirect effect of light on the growth medium, a direct effect of light on the pyocyanin molecule or a direct effect of light on *P. aeruginosa*. When uninoculated growth medium was pre-irradiated with broad-spectrum light and subsequently inoculated with *P. aeruginosa*, cultures grown in the dark on unirradiated and pre-irradiated media had similar concentrations of pyocyanin (Table 1). Thus, the effect of light on pyocyanin was not due to an irreversible, indirect effect of light on the growth medium. Similar results were obtained with 371 nm light at $300 \mu\text{W cm}^{-2}$ (data not presented). However, when plates with dark-grown *P. aeruginosa* had their micro-organisms removed and were subsequently exposed to broad-spectrum light (Table 1) or 371 nm light at $300 \mu\text{W cm}^{-2}$ (data not presented), the pyocyanin concentration decreased. Thus it appears that light has a direct effect on pyocyanin after its release from bacteria.

Photoinactivation of pyocyanin extracts

To confirm that light affected pyocyanin directly, aqueous extracts of pyocyanin were exposed to broad-spectrum light and the photoinactivation of the pigment was observed as a function of time (Fig. 2). Photoinactivation of pyocyanin appeared to follow first-order kinetics. Pyocyanin had a half-life of about 36 h. Incubation of the colourless photoproduct(s) in the dark for up to 4 d did not result in recoloration. Spectra of pyocyanin and its photoproduct(s) at various times (arrowed in Fig. 2) during the course of photoinactivation are shown in Fig. 3.

DISCUSSION

The results presented here show that exposure of *P. aeruginosa* cultures to light can produce changes in observable pigmentation. Pyorubrin and pyoverdine concentrations were either unaffected or only marginally affected by the lighting conditions tested, whereas the pyocyanin concentration decreased drastically. Subsequent studies indicated a direct

Fig. 2. Inactivation of pyocyanin extract by light. Pyocyanin, extracted as described in Methods, was thinly layered in Pyrex Petri dishes (6 ml per 15×60 mm dish) and exposed to broad-spectrum light at $2400 \mu\text{W cm}^{-2}$. The Petri dishes transmitted 90% of the incident light above 345 nm. At various times, samples were removed and their absorbance at 660 nm was determined.

Fig. 3. Spectra of pyocyanin and its photoproduct(s). At various times during the photoinactivation of pyocyanin extracts (see Fig. 2a to f), visible and ultraviolet spectra were recorded using a Perkin Elmer model 570 spectrophotometer.

effect of light on the pigment, its inactivation resulting in the production of colourless photoproduct(s). The initial observations that dark-grown cultures produced blue–purple coloured plates while light-grown cultures produced red agar are thus explained by the fact that plates kept in the dark contain pyocyanin (blue) and pyorubrin (red) pigments whereas plates exposed to light contain pyorubrin (red) and the photoproduct of pyocyanin (colourless). Both light- and dark-grown cultures produce pyoverdine (yellow) which is too weak in colour to be visually detectable above the blue–purple and red backgrounds. The above findings also provide a likely explanation for the frequent observation in older literature that *P. aeruginosa* cultures show the gradual appearance of reddish brown colour when allowed to age in the laboratory exposed to ‘air’ (see Leonard, 1924).

Studies on pyocyanin both *in situ* (see Fig. 1) and in extracts (data not presented) showed that exposure to 371 nm (near ultraviolet) and 420 nm (violet) light resulted in the photoinactivation of this pigment, whereas 550 nm (green), 660 nm (red) and 740 nm (far red) light had little or no effect. Additional studies with pyocyanin extracts showed that this pigment is also strongly photoinactivated by light of wavelengths shorter than 371 nm, specifically 254, 313 and 365 nm (data not presented). The shorter the wavelength of ultraviolet light, the more rapidly the inactivation occurred for any given intensity. At present, the photoproduct(s) of pyocyanin have not been isolated and the chemical structure(s) remain unknown. However, on the basis of the wavelengths of light absorbed, the nitrogen-containing tricyclic structure of pyocyanin, and the downward shift in ultraviolet spectra after irradiation (see Fig. 3), it seems most likely that the N-containing ring is the site of disruption by light.

As noted above, pyocyanin is sensitive to photoinactivation by light of wavelengths shorter than 371 nm. Elliott (1958) reported that pyoverdine extracts in neutral solution are also inactivated by ‘short wavelength’ ultraviolet light, with a resultant decrease in fluorescence. However, since no loss of fluorescence was observed in the present study, it appears that photoinactivation of pyoverdine does not occur *in situ* under the conditions tested.

The effect of light on pyocyanin is of interest for both scientific and clinical reasons. Although pyocyanin *in situ* in agar plates is reasonably well protected from photoinactivation by normal intensities of laboratory light (100 to 200 $\mu\text{W cm}^{-2}$) and several days of continual exposure are needed in order to produce a visible colour change, extracted pigment is much more vulnerable. Care should be taken to prevent excessive exposure of pyocyanin extracts being used for quantitative or spectral studies to light, especially below 500 nm. The use of pyocyanin production as a rapid identifying characteristic of *P. aeruginosa* is another good reason for continuing the usual practice of growing clinical cultures in the dark.

We would like to thank Dr Charles Botticelli and Mr Joseph Weidenheimer for critical evaluation of this manuscript, and Ms Barbara Schaller for expert technical help.

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