Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol16-issue4-fulltext-7 *RESEARCH ARTICLE*

Prodigiosin found in *Serratia marcescens* y2 initiates phototoxicity in the cytomembrane

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Received November 12, 2012 / Accepted May 28, 2013
Published online: July 15, 2013

Abstract

Background: Light can be absorbed by bacterial pigment and affects its growth. Prodigiosin is a red pigment found in various bacterial species. The purpose of this study was to investigate the impacts of light on prodigiosin production, biomass formation, and membrane integrity of *Serratia marcescens* y2.
Results: *S. marcescens* y2 grew better and produced more intracellular prodigiosin in darkness than in illumination. The pigment leakage ratio from cells was detected more in light than in darkness conditions. Ethidium bromide uptake assay could visually prove the prodigiosin-related loss of membrane integrity under illumination. A higher concentration of malondialdehyde (MDA) was detected in light-treated culture than in darkness. Tests of different light treatments (red, yellow, blue and green) showed that the maximum extracellular pigment and the minimum biomass formation and intracellular pigment were obtained in green light.
Conclusions: Prodigiosin could absorb light, and then initiate phototoxicity damage of the cytomembrane.

Keywords: ethidium bromide, homogeneous light, malondialdehyde, red pigment.

INTRODUCTION

Serratia marcescens, a gram-negative, rod-shaped bacterium, can be isolated from ubiquitous environment. The organism is involved in multiple infectious diseases, such as keratoconjunctivitis, arthritis, bacteremia, respiratory infections, urinary tract infections, etc. (Pérez-Tomás and Viñas, 2010). Although *S. marcescens* isolated from its environment produces the red, cell-associated pigment prodigiosin, the majority of clinical isolates are rarely pigmented (Hejazi and Falkiner, 1997).

Prodigiosin is typically a secondary metabolite, appearing only in the later stages of bacterial growth. This red pigment can be produced by *S. marcescens* and other microorganisms, such as *Zooshikella rubidus*, *Vibrio* sp., *Streptomyces griseoviridis*, and *Hahella chejuensis* (Lewis and Corpe, 1964; Song et al. 2006; Huh et al. 2007; Alihosseini et al. 2008; Kawasaki et al. 2008; Kim et al. 2008; Borić et al. 2011; Lee et al. 2011). Prodigiosin has great potential for clinical and environmental application owing to its reported characteristics of having anti-fungal, anti-bacterial, anti-protozoal/anti-malarial, immunosuppressive, and antiproliferative activity (Williamson et al. 2005). Prodigiosin has recently been considered an effective biological control agent against harmful marine algae (Kim et al. 2008; Venil and Lakshmanaperumalsamy, 2009).

The production of prodigiosin by *S. marcescens* is influenced by numerous factors including inorganic phosphate availability, medium composition, temperature, pH, and natural components (Solé et al. 1997; Hardjito et al. 2002; Iranshahi et al. 2004; Wei et al. 2005). Specifically, light has an inhibiting and destructive effect on the production and stability of prodigiosin, and prodigiosin is considered to be capable of storing visible light energy (Ryazantseva et al. 1995; Someya et al. 2004). But *S. marcescens* TKU011 was reported to grow under illumination in shaking culture conditions, where it exhibited higher prodigiosin productivity than under dark conditions (Wang, 2012). However, the effect of light was not well illustrated. The objective of this study is to explore the effects of light on the production of prodigiosin and biomass formation of *S. marcescens* y2.

MATERIALS AND METHODS

Microorganisms and materials

S. marcescens y2 was isolated from the leaves of wheat in the city of Nanjing, China. It was maintained and cultured in PSA medium (200 g potato, 20 g sucrose, and 15 g agar in 1,000 mL sterile distilled water). Varian Cary 100 Conc UV-Visible Spectrophotometer (Agilent Technologies) was used for absorbance studies. Agilent 1260 infinity with a diode array detector (Agilent Technologies) was used for high performance liquid chromatography (HPLC) analysis. 1,1,3,3-tetraethoxypropane (TEP) and dinitrophenylhydrazine (DNPH) were supplied by Sigma-Aldrich Com Limited. Organic solvents were chromatographic grade. All other chemicals were of analytical grade.

Light treatment

The isolates were inoculated in liquid seed culture medium (PS) and cultured in darkness at 28ºC for 24 hrs, and then a 100 µL of the cultures were spread uniformly over the surface of solid PSA in 9-cm Petri dishes. During incubation, the cultured bacteria were kept at a temperature of 28ºC under different light treatments, including under continuous dark and white light, and followed by red, yellow, blue and green light, respectively. White light was provided with 40 watt white fluorescent lamps positioned at a height of 20 cm (approximately 4,000 lux) above the cultures. Different light source were created by coloured glass paper (Velmurugan et al. 2010).

After 24 hrs of light treatment, biomass was scraped off plates, and resuspended in a final volume of 5 mL sterile water for the next tests.

Pigment extraction and analysis

The suspension was centrifuged at 10,000 rpm for 20 min at 4ºC. The supernatant was transferred to a fresh tube before acquiring the extracellular pigment concentration by HPLC. The deposit after centrifugation that remained in the tube was extracted twice with ethanol (final volume, 5 mL), collected and analyzed as intracellular pigment. Extracts were filtered (0.2 µm) immediately before HPLC analysis. Chromatographic separation was carried out using a C18 column for reverse phase chromatography (Eclipse plus C18, 4.6 x 100mm), with a flow rate of 0.8 ml min^{-1} and an injection volume of 10 µL. The solvents used were 0.5 M phosphate buffer at pH 2.0, methanol and water (10:75:15, v/v). The wavelength for detection was 535 nm. The concentration of prodigiosin was determined by measuring the absorbance, and then calculated using a standard correlation curve between absorbance and the dry weight of prodigiosin. The standard prodigiosin used in this research was prepared through silica gel and reverse-phase chromatography. The pigment leakages were calculated by the ratio of extracellular to intracellular pigment.

Biomass estimation

After pigment extraction, bacterial pellets were washed twice with deionized water, and then dried at 105ºC for 12 hrs until weight remained constant, and the biomass was weighted.

Ethidium bromide uptake

1 mL volumes of light-treated cell suspensions of *S. marcescens* y2 were incubated with 100 µL of ethidium bromide solution (1 mg/ml) at 4ºC for 2 hrs with shaking. Cells cultured in darkness served as controls. Cells were washed and resuspended in deionized water, and a drop of each suspension was examined with an inverted fluorescence microscope (Eclipse TS100-F, Nikon Corp., Japan) for red fluorescence. In a parallel experiment, cells of non-pigment producing *S. marcescens* mutants with identical treatments were used to evaluate the role of prodigiosin. The percentage of red fluorescence cells was calculated in 5 counting fields.

Malondialdehyde (MDA) HPLC assay

After incubation on the PSA medium under different light treatments, the cells were centrifuged at 10,000 rpm for 20 min, and the supernatants were discarded. The pellets were resuspended in 5 mL aliquots of 1.15% KCI, and then homogenized for sample preparation. A non-pigment producing (mutant) strain under similar growth conditions was used as control. The MDA standard curve and HPLC analysis were prepared by the methods of Tukozkan (Mateos et al. 2005; Tukozkan et al. 2006). In the experiment, the analytical column was Eclipse plus C18, 4.6 x 100 mm. The results were evaluated by the standard curve (0.01, 0.02, 0.04, 0.08, 0.16 nmol/mL) and expressed as nmol/mg protein, suing the Bradford method to determine the protein concentration of bacterial cells.

Statistical analysis

A one-way ANOVA was applied to determine the significant difference ($P < 0.05$), and Fisher's protected LSD test was used to separate means ($P < 0.05$).

RESULTS

Pigment production and biomass formation of *Serratia marcescens* y2

Both intra and extracellular pigment shared the same retention time (data not shown). Figure 1 shows the effects of light on pigment production of *S. marcescens* y2. The intracellular pigment decreased when *S. marcescens* y2 was cultured under white light. The biomass formation of *S. marcescens* y2 was reduced by white light. The ratio of pigment (extracellular and intracellular) to the biomass was 0.0012 under white light conditions, showing no significant difference with that of 0.0016 in darkness ($p < 0.05$, n = 3). However, the ratio of extracellular pigment to the biomass was 0.0031 under white light conditions, which was significantly higher than that of 0.0023 in darkness ($p < 0.05$, n = 3). Additionally, the ratio of pigment leakage was 0.36 under white light conditions, also significantly higher than that of 0.16 in darkness ($p < 0.05$, n = 3). These results suggested that more pigment produced by y2 was released into extracellular space when cultured under white light than in darkness.

Membrane integrity assay

To assess the effects of light on the permeability of *S. marcescens* y2, bacterial cells were incubated on a PSA medium in light or dark conditions. As assessed by the emission of red fluorescence after ethidium bromide staining, the prodigiosin-induced permeabilization of cell membrane of *S. marcescens* y2 treated with light could be identified. Cells of non-pigmented mutants (y^-) cultured in light and darkness conditions contain similar percentages of red fluorescence cells. However, wild types (y^+) have a significantly higher percentage of red fluorescence cells in light than in darkness (Figure 2).

MDA analysis

Quantification of MDA was carried out by using a standard curve relating HPLC integrated peak areas to concentrations of acid hydrolyzed TEP in the range of 0.01-0.16 nmol/mL ($y = 369.85x – 0.377$; $R^2 = 0.9984$; y:peak area; x:MDA concentration). Figure 3 showed that the HPLC elution profiles of MDA-DNPH complex at t = 3.89 min as absorbed at 310 nm. The MDA concentration in extracts from light-

treated culture was 0.045 ± 0.008 nmol/mg (n = 3). The concentrations of MDA from culture in darkness and non-pigmented mutants in light-treated culture were too low to be detected by the HPLC analysis.

Effects of coloured lights

Pigment production was significantly different in red, yellow, blue, and green lights (Figure 4). The maximum intracellular pigment production was obtained in red light, and the minimum was obtained in green light. For extracellular pigment, the maximum production was in green light, and was higher than in red lights. The ratios of pigment leakages were 0.16 and 0.31 when incubated in red and green light, respectively (data not shown). Our observation suggested that more pigment was secreted from bacterial cells when exposed in green light. Meanwhile the minimum biomass formation was observed in green light, which indicated green light would cause strong negative effects on the growth of *S. marcescens* y2.

DISCUSSION AND CONCLUDING REMARKS

Prodigiosin production by microbes depended on culture factors including illumination (Venil and Lakshmanaperumalsamy, 2009; De Araújo et al. 2010). This study reproduced the inhibitory effect of light on intracellular prodigiosin production of *S. marcescens* y2 as reported previously (Ryazantseva et al. 1995; Ryazantseva et al. 2012). Prodigiosin was taken as a hydrophobic component linked to the inner membrane and insoluble in water (Viñas et al. 1983; Paruchuri and Harshey, 1987). In this study, the ratios of pigment leakages were higher in light than in darkness and combined with less biomass formation obtained in light suggested the loss of the bacterial membrane integrity and the deleterious cytoplasmic effects of light (Figure 1).

In this paper, typical phototoxicity arises when the pigmented bacterium is exposed in white light (Björn, 2002). As assessed by the uptake of ethidium bromide, which is characteristic of the loss of membrane integrity, a higher percentage of *S. marcescens* y2 (y^+) cells loaded with the fluorescent dye was found in light than in darkness. However, light-treated cells of the non-prodigiosin strain (y^-) could not increase the percentage of stained cells by fluorescence dye. Therefore, this result showed the critical role of prodigiosin during light stress. In this study, due to the low permeability of the gram-negative bacterial cell wall, the staining process with ethidium bromide was prolonged to 2 hrs, at 4ºC to acquire clearly visible red images under the fluorescent microscope.

It is generally accepted that lipid peroxidation is correlated with the ultimate disintegration of membrane integrity and pigment leakage. Prodigiosin had been reported to induce lipid peroxidation in the presence of Cu (II) and damage cytoplasmic membrane in its cytotoxicity (Subramanian et al. 2007). There for the MDA assay was performed with both strains of pigmented wild type (y^+) and non-pigmented mutant (y^-). Malondialdehyde is one of the most studied secondary products of membrane lipid peroxidation and is quantified as a measure of oxidative stress both *in vitro* and *in vivo*. There is a widely held belief that prodigiosin is attached to the inner membrane (Venil and Lakshmanaperumalsamy, 2009). One would therefore reasonably expect to identify the trace of MDA, a good marker for the injured membrane in the bacterial cell cultured in light or dark. The HPLC method after derivatization with DNPH provided a sensitive and accurate assessment of lipid peroxidation. In this paper, the HPLC analysis resulted in 0.045 ± 0.008 nmol/mg protein of total MDA in the cell from the pigmented strain in light. However, there was no MDA detected from the pigmented strain in darkness or the non-pigmented mutant in light. We propose that the prodigiosin, linked to the bacterial plasma membrane, could absorb visible light and generate reactive oxygen. The pigment and the cellular membrane were attacked by reactive oxygen. Subsequent lipid peroxidation damaged the integrity of the cellular membrane and led to the accumulation of extracellular pigment and a decrease in biomass.

It is interesting to discuss some roles of prodigiosin in the pigment production of *S. marcescens* y2, cancer cells, and other potential targets. Light is a complex environmental factor influencing the function of prodigiosin. Previous studies revealed that the cytotoxicity of prodigiosin analogues on HL-60 cancer cells was photoinduced (Park et al. 2007), and supposed that prodigiosin-based pigments can trigger photochemical DNA damage. Due to photosensitivity of prodigiosin, its production in *S. marcescens* decreased under continuous illumination with visible light. It was suggested that visible

light acts directly on the production of the pigment by culture (Ryazantseva et al. 1995). Among the four light treatments, culture in green light (about 500-565 nm) presented the maximum extracellular pigment and the minimum intracellular pigment and biomass formation, which was the opposite of that in the red light. These results indicated prodigiosin, the red pigment, was the absorber of visible light and the sponsor of phototoxicity.

ACKNOWLEDGMENTS

We would like to thank Alison Cassidy at the University of British Columbia for her assistance with English language and grammatical editing of the manuscript.

Financial support: The Key Technologies Program of Henan Province (102102310373), the Natural Science Foundation of Henan Educational Committee (2010C550003), the Foundation for Key Teacher of Henan Educational Committee (2010GGJS-287), Luohe Science and Technology Development Funds, and the Research Program of Henan Fundamental and Advanced Technology (132300410173) provided financial supports for this study.

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How to reference this article:

WANG, F.; LUO, H.; SONG, G.; LIU, C.; WANG, J.; XU, J.; SU, X. and MA, X.Y. (2013). Prodigiosin found in *Serratia marcescens* y2 initiates phototoxicity in the cytomembrane. *Electronic Journal of Biotechnology*, vol. 16, no. 4. http://dx.doi.org/10.2225/vol16-issue4-fulltext-7

Figures

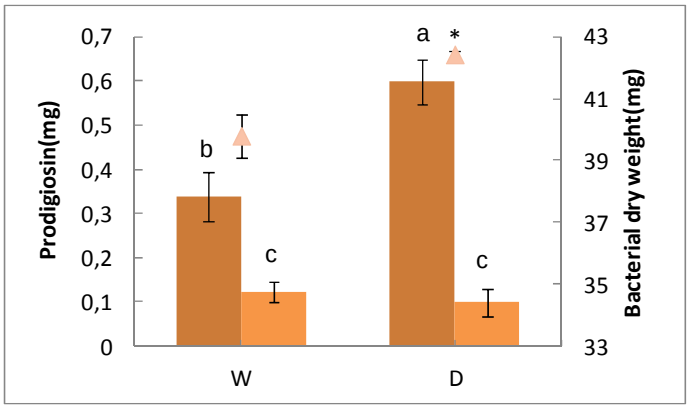

Fig. 1 Effects of white light on intra and extracellular prodigiosin accumulation and biomass of *S. marcescens* y2 on PSA medium (9-cm Petri dish). Cultures were incubated at 28ºC for 24 hrs in continuous white (W) light or darkness (D). Intracellular pigment was extracted from collected bacterial cell (dark columns). Extracellular pigment was harvested by centrifugation of suspension (light columns). Columns marked with different letters are significantly different at $p < 0.05$ using Fisher's protected LSD test (n = 3). Bars on the columns represent standard error. Biomass was measured by dry weight of collected bacterial cell (triangle). Data represent average values and standard error (n = 3). Statistical significance between biomass of treated bacteria are indicated as * $p < 0.05$.

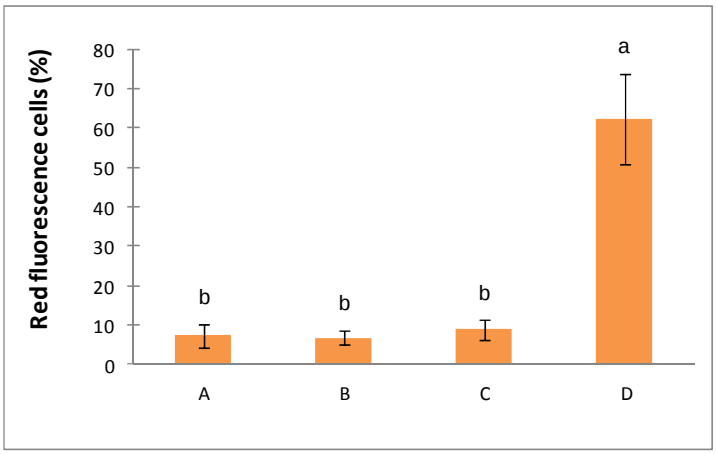

Fig. 2 Percentage of red fluorescence cells shows the results of ethidium bromide uptake of *S. marcescens* y2. Percentages of red fluorescence cells in pigmented cells ($y2^+$) in darkness and non-pigmented mutation ($y2^-$) in both light and darkness indicated no differences. However pigmented cells in light showed a significantly higher percentage of red fluorescence cells in light. A: $y2^-$ in darkness; B: $y2^-$ in light; C: $y2^+$ in darkness; D: $y2^+$ in light. Columns marked with different letters are significantly different at $p < 0.05$ using Fisher's protected LSD test (n = 5). Error bars on the columns represent standard error.

DOI: 10.2225/vol16-issue4-fulltext-7

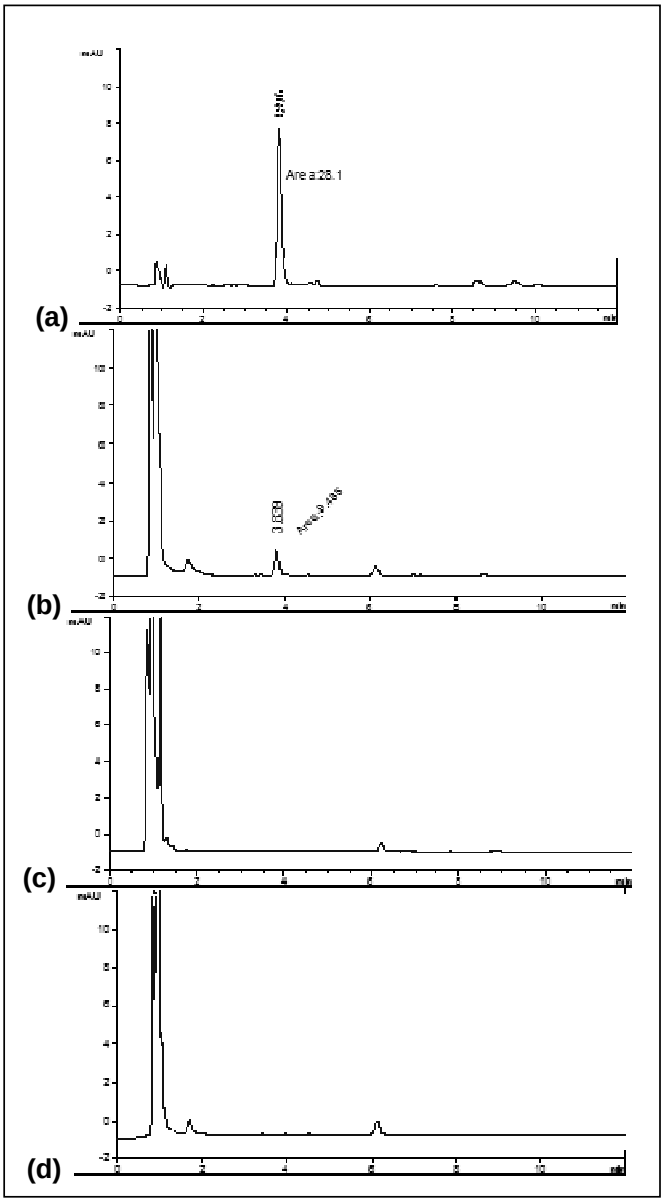

Fig. 3 HPLC chromatograms of MDA-DNPH complex from MDA standard, bacterial cell of *S. marcescens* y2 cultured on PSA medium with and without light treatment after DNPH derivatization. A 10-µl aliquot of sample was injected onto a C-18 reverse-phase column (3.5 µm, 4.6 x 100 mm). Mobile phase: acetonitrile/water/acetic acid 36/62/0.2 (v/v); flow rate, 1 mL/min; detector, UV-vis, 310 nm. (a) 0.08 nmol/mL MDA provided by acid hydrolysis of TEP in 1% sulphuric acid and incubation for 2 hrs at room temperature. (b) MDA from culture under light treatments; (c) MDA from bacterial cell cultured in darkness; (d) MDA from non-pigmented mutant cultured under light treatments.

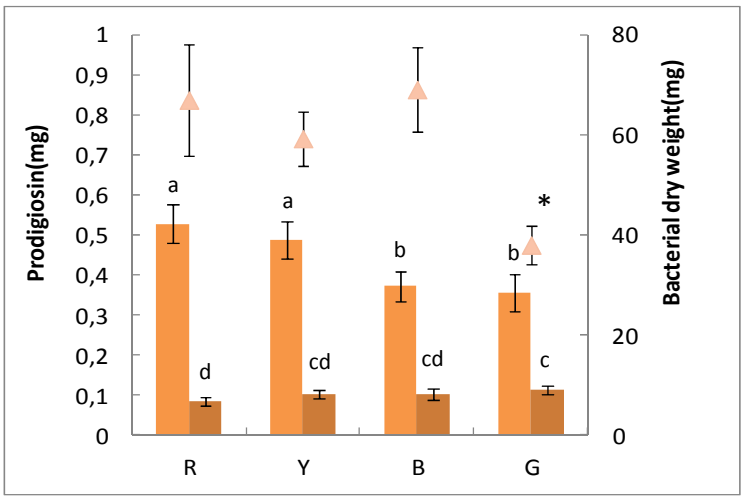

Fig. 4 Effect of different light sources on intra- and extracellular prodigiosin accumulation and biomass formation of *S. marcescens* y2 on PSA medium (9-cm Petri dish). Cultures were incubated at 28ºC for 24 hrs in continuous red (R), yellow (Y), blue (B), or green (G) light. Intracellular pigment was extracted from collected bacterial cells (dark columns). Extracellular pigment was harvested by centrifugation of suspension (light columns). Columns marked with the same letters are not significantly different at $p < 0.05$ using Fisher's protected LSD test (n = 3). Error bars on the columns represent standard error. Biomass was measured by dry weight of collected bacterial cell (triangle). Data represent average values and standard error (n = 3). Statistical significance between biomass of treated bacteria are indicated as * $p < 0.05$.

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