Influence of environmental factors on musty odor production by *Streptomyces coelicolor* A3(2)

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Abstract:

The occurrence of the offensive musty odor has become a serious problem for water supplies and fisheries. Actinomycetes are known to produce geosmin which is responsible for the musty odor. However, it is still unclear what environmental factors control Actinomycetes geosmin production. In this study, we conducted culture experiments under starvation condition and light irradiation to elucidate the factors that influence production of geosmin. As a result, the geosmin concentration started to decline from mid to late M2 cell cycling stage by addition of filter sterilization liquid culture media that was pre-incubated *S. coelicolor* A3(2) under starvation condition. Furthermore, two well-characterized antibiotics (ACT and RED) concentrations were also decreased. These results suggest that the cultured liquid media contains some signal molecules that are associated with geosmin production. On the other hand, to irradiate specific wavelengths light which was white (multiple wavelength), blue (peak wavelength: 460 nm), green (peak wavelength: 519 nm), or red (peak wavelength: 792 nm) induced geosmin production activity compared with dark condition (white: 27.5 μM > blue: 17.7 μM > green: 13.0 μM > red: 7.9 μM > dark: 2.3 μM). It is strongly suggested that the lights play a key role in the induction of geosmin production activity by *S. coelicolor* A3(2).

Key words

Geosmin production activity, actinomycetes, starvation condition, light irradiation

1. Introduction

In recent years, the occurrence of musty odor concerns originated from microorganisms in lakes, rivers, and reservoirs for water supplies has been reported in all over the world (Juttner and Watson 2007). These problems threaten quality of water supply and industry. The typical musty odor compounds, 2-methylisoborneol (MIB) and geosmin, are secondary metabolites produced by some filamentous cyanobacteria and actinomycetes in aquatic environment (Izaguirre et al. 1982, Sugiura et al. 1998, Sugiura and Nakano 2000, Zaitlin and Watson 2006). These compounds can be perceived by human being at very low concentration level (ng/L) (Young et al. 1996) and it is ineffective to remove by conventional water supply treatment processes such as coagulation, sedimentation, filtration and chlorination (Bruce *et. al.* 2002). As methods of removing these compounds, there are advanced water treatment techniques such as active carbon process, ozone oxidation process, and these combination process (Srinivasan and Sorial 2011). Although the advanced water treatment process is effective, it is too expensive to be applied in all municipalities. Therefore, establishment of the cheap and effective removal methods is essential for water supply reservoirs *in situ*. However, it is still unclear what environmental factors control the production of these compounds in aquatic environments.

Actinomycetes, which form pellets under submerged condition, produce secondary metabolites with growing pellets. One of the reasons for this is that cell death

process take place in the center of the pellets, during the inside pellet cell growing (Manteca et al. 2008). Altogether, it is considered that starvation conditions such as oligotrophic and hypoxic can induce cell lysis in the center of pellets, and then signal molecules are released efficiently to the extracellular environment. One of the molecules considered to be released by this phenomenon was *Streptomyces coelicolor* γ-butyrolactone (SCBs). SCBs is the γ-butyrolactone bacterial hormones, and it regulates antibiotic production and morphological differentiation (both positive and negative), and this hormone is active even at nanomolar concentrations (Takano et al. 2000, Takano et al. 2001, Hsiao et al. 2009). It was reported that the two antibiotics, actinorhodin (ACT) and undecylprodigiosin (RED) are regulated by this bacterial hormone, and the production of these two antibiotics are started after cell death process in the pellets (Manteca et al. 2008).

Geosmin is terpenoids as well as carotenoid. It is previously reported that blue light controls carotenoid production in actinomycetes (Takano et al. 2005, Takano et al. 2006). Furthermore, isopentenylpyrophosphate (IPP) is common precursor of geosmin and carotenoid. Therefore, it is assumed that light influences on geosmin production by actinomycetes.

Based on these backgrounds, we examined what environmental factors influence on production of geosmin by actinomycetes. Here we reported that the results of culture experiments under the starvation condition and light irradiation using *Streptomyces coelicolor* A3(2).

2. Methodology

2.1 Bacteria strain

Streptomyces coelicolor A3(2), a strain whose genome has been fully sequenced (Bentley et al. 2002), was used in this study.

2.2 Starvation condition experiment

S. coelicolor A3(2) was pre-cultured at 28°C for 2 days in 100 ml YMPD medium (2 g yeast, 2.2 g meat extract, 4 g bacta pepton, 2 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, 1 g glucose, pH 7.2, per litter) with reciprocal shaking at 120 rpm. Total cells were harvested by centrifugation (7,500 × *g*, 3 min) and were washed twice with BS medium without carbon source (BS negative; 2 g KNO_3, 0.5 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, pH 8.0 per litter). They inoculated into 1 l Erlenmeyer flasks with silicon rubber stopper, containing 400 ml of dispersed BS negative medium (2 g $(NH_4)_2SO_4$, 2 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 0.05 g $FeSO_4 \cdot 7H_2O$, pH 8.0, per litter). After 2 days cultivation with rotary shaking at 120 rpm under 28°C, the liquid culture was filtrated by 0.2 μm pore size polycarbonate filters (Whatmann). *S. coelicolor* A3(2) in the cultured medium was harvested and washed as previously described. Transfer the washed pellets to sterilized eppendorf tube and homogenized with 1 mm glass beads, it was inoculated to give a final pellet count of 1×10^7 pellet/ml in dispersed BS medium (20 g starch, 5 g mannitol, 2 g $(NH_4)_2SO_4$, 2 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 0.05 g $FeSO_4 \cdot 7H_2O$, 0.5 g yeast, 0.5 g agar, pH8.0, per litter) (Hobbs et al. 1989). All the mediums were adjusted pH with 3 M NaOH before autoclaving. *S. coelicolor* A3(2) was grown in 1 l Erlenmeyer flasks with silicon rubber stopper, containing 400 ml dispersed BS medium at 28°C for 12 days with reciprocal shaking at 120 rpm. Twenty ml of filter sterilization liquid pre-culture medium was added at the start of inoculation (0 h). *S. coelicolor* A3(2)

which was incubated without the addition of filter sterilization liquid pre-cultured medium was used as control. We measured growth curve, geosmin concentration, and antibiotics (ACT and RED) concentration during the incubation.

2.3 Light irradiation condition experiment

S. coelicolor A3(2) was pre-cultured, harvested, and washed as previously described. After homogenized by 1 mm glass beads, 100 μl of pellets were spread on SFM agar plates (20 g soy flour, 20 g mannitol, 15 g agar, per litter). Plates were cultured for 7 days at 28°C under light or dark condition. Under light condition, while light was illuminated at approximately 130 lx onto the plates. The same lamp, covered with a blue, green, or red light filter, was used for illumination of blue (peak wavelength: 460 nm), green (peak wavelength: 519 nm), or red (peak wavelength: 792 nm) light, respectively. We measured geosmin concentration at the end of incubation.

2.4 Growth curve

Optical densities during the incubation were determined at 450 nm using UV-VIS spectrophotometer against appropriate media blanks (Hobbs et al. 1989).

2.5 Analysis of geosmin under starvation condition

Gesmin concentration in liquid cultures were analyzed by adding a 1.0 ml of n-hexane to 5.0 ml of culture sample. The mixture was stirred for 30 min, and then centrifuged at 800 × *g* and 60 min to separate the n-hexane layer. The n-hexane layer was carefully collected and was filtered through a column of Na_2SO_4 in a Pasteur pipette for removing humidity (Jensen et al. 1994). A 1.0 μl portion of the extract was analyzed for geosmin concentration by GC-MS 2010 plus (Shimadzu Co Ltd. Japan).

2.6 Analysis of geosmin under light irradiation condition

After 7 days cultivation, 5.0 ml of methanol was directly added to each plate, and the plates were kept at room temperature for 30 min. A 1.0 ml methanol extract was collected in glass tube and added 2.0 ml of n-hexane. The mixture was stirred for 30 min and then centrifuged at 800 × *g* and 60 min to separate the n-hexane layer. The n-hexane layer was carefully collected and was filtered through a column of Na_2SO_4 in a Pasteur pipette (David and He 2006, Komatsu et al. 2008). A 1.0 μl portion of the extract was analyzed for geosmin concentration by the GC-MS.

2.7 GC-MS analysis condition

The GC-MS conditions were as follows: 50°C for 1 min, 15°C/min to 250°C, final temperature hold for 5 min; Rix-5MS column, 30 m × 0.25 mm × 0.25 μm (Restek); vaporizing chamber temperature 230°C; interface temperature 230°C; ion source temperature 200°C.

2.8 Analysis of antibiotics

ACT and RED concentrations were determined according to previously report (Ryu et al. 2006). The same operations were performed on appropriate media as blanks.

3. Results and Discussion

3.1 Starvation condition experiment

Both control and sample which was added the filter sterilized pre-culture medium that was pre-incubated *S. coelicolor* A3(2) in starvation condition showed plateau

growth from 48 to 72 h, and then growth rate turned positive again (Figure 1a). Therefore, it was suggested that 0 to 48 h, 48 to 72 h, and after 72 h were classified to M1 stage, growth arrest phase, and M2 stage, respectively.

Geosmin concentration in the control showed maximum concentration (2.9 nM) at 24 h. Then it kept approximately constant value. Geosmin concentration in the sample showed maximum concentration (3.0 nM) at 24 h, as well as the control. It was noteworthy that geosmin concentration decreased from mid to late of M2 stage (144 to 288 h), and it showed 0.06 nM at 288 h (Figure 1b).

ACT production in the control was observed after 144 h. The concentration gradually increased, showed maximum (70.4 μM) at 288 h. On the other hand, significant ACT concentration increase in the sample was not observed after 144 h. The maximum concentration was 6.0 μM at 288 h (Figure 1c). RED concentration in both control and the sample began to increase after 144 h, and the sample showed slightly lower concentration than control. The maximum concentration of control and the sample were 0.35 μM and 0.20 μM at 288 h, respectively (Figure 1d).

Our results demonstrated that the production activity decline of geosmin, ACT, and RED were measured at the pre-cultured medium adding condition. These results suggested that the pre-cultured medium under the starvation condition possess some signal molecules that were associated with geosmin and antibiotics production for *S. coelicolor* A3(2). It is considered that SCBs production period is consistent with the time when cell death process takes place in the center of the pellets (Manteca et al. 2008). Furthermore SCBs induce antibiotic production even at very low concentration and inhibit the antibiotic production at high concentration (Takano et al. 2001). Therefore, it was estimated that the pre-cultured medium incubated in starvation condition included large amounts of SCBs, and geosmin production was controlled by SCBs. The next step, we examine in more detail the relationship between geosmin production and SCBs.

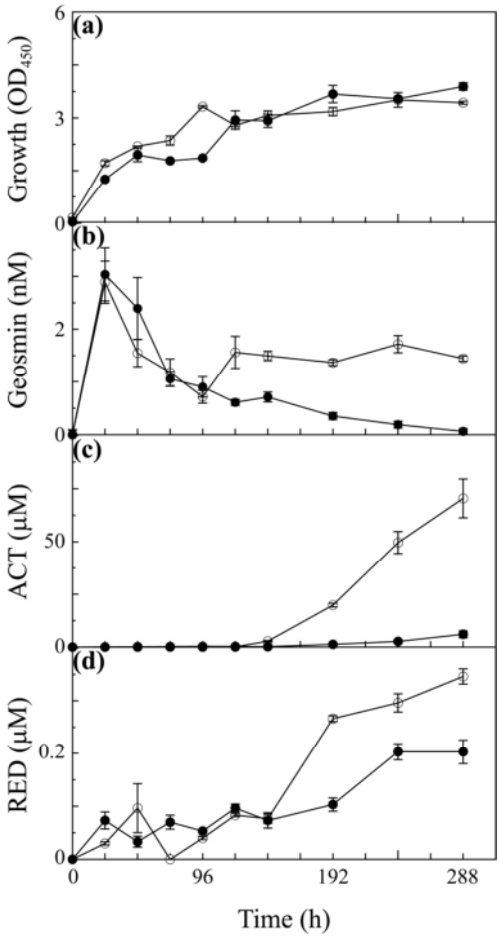

Figure 1. Effect of filter sterilization liquid culture. (a) Growth curve. (b) Concentration of geosmin. (c) Concentration of ACT. (d) Concentration of RED. Solid circles shows sample (added filter sterilization liquid culture at 0 day); Open circles show control (without filter sterilization liquid culture). Error bar indicate standard deviations from three independent experiments.

3.2 Light irradiation condition experiment

Figure 2 showed the geosmin concentration after 7 days incuvation under light or dark condition. The

concentration under dark condition was 2.3 μM, whereas under white light condition showed tenfold higher concentration (27.5 μM). Then we investigated influence of geosmin production activity under different wavelength conditions. Geosmin concentration under red, green, and blue light showed 7.9 μM, 13.0 μM, and 17.7 μM, respectively. These results strongly suggested that the lights played a key role in the induction of geosmin production activity by *S. coelicolor* A3(2).

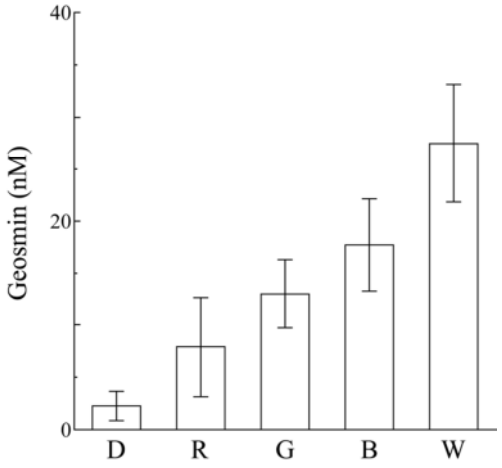

Figure 2. Effect of light irradiation to production of geosmin. D: Dark, R: Red, G: Green, B: Blue, W: White. Error bar indicate standard deviations from three independent experiments.

Carotenoids production of *S. coelicolor* A3(2) is induced by blue light irradiation (Takano et al. 2005, Takano et al. 2006). Furthermore, IPP is common precursor of geosmin and carotenoid. We therefore hypothesized that blue light condition decreases geosmin production activity, because carotenoids biosynthesis pathway is activated under this condition. However S. *coelicolor* A3(2) produced high concentration of geosmin to irradiate lights including blue light. Consequently, it was suggested that light activates the entirety terpenoids biosynthesis (date not shown). Furthermore, white light condition leaded to the highest geosmin concentration, and the specific wavelengths light that was blue, green, or red also showed higher concentration compared with dark condition (white: 27.5 μM > blue: 17.7 μM > green: 13.0 μM > red: 7.9 μM > dark: 2.3 μM). Thus it was indicated that various wavelengths of light would induce production of geosmin by *S. coelicolor* A3(2).

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