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Use of red autofluorescence for monitoring prodiginine biosynthesis

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ABSTRACT

Prodigiosin-like pigments or prodiginines (PdGs) are promising drugs owing to their reported antitumor, antibiotic, and immunosuppressive activities. These natural compounds are produced by several bacteria, including *Streptomyces coelicolor* and *Serratia marcescens* as most commonly studied models. The bright red color of these tripyrrole pigments made them excellent reporter molecules for studies aimed at understanding the molecular mechanisms that control secondary metabolite production in microorganisms. However, the natural red fluorescence of PdGs has only been rarely used as a biophysical parameter for detection and assessment of PdG biosynthesis. In this work, we used *S. coelicolor* in order to exemplify how intrinsic red fluorescence could be utilized for rapid, low-cost, sensitive, specific and accurate semi-quantitative analyses of PdG biosynthesis. Additionally, and contrary to the colorimetric-based approach, the fluorescence-based method allows in situ spatio-temporal visualization of PdG synthesis throughout a solid culture of *S. coelicolor*. As PdG production is related to cell differentiation, their red autofluorescence could be exploited, by means of confocal microscopy, as a natural marker of the entrance into a crucial developmental stage in the course of the *S. coelicolor* life cycle.

1. Introduction

Intrinsic fluorescence provides reliable, high-speed (with minimal sample preparation), and low-cost methodologies for the detection and identification of microorganisms as well as for the evaluation of their metabolic status (Ammor, 2007). To cite only a few of them, autofluorescence-based approaches have been successfully applied to morphological studies of microalgae (Tang and Dobbs, 2007) and to species determination (Bhatta et al., 2006; Leblanc and Dufour, 2002). Additionally, specific excitation/emission spectra have been suggested as means to uncover the presence of viable but non-cultivable (VBNC) microorganisms otherwise not detectable by conventional techniques (Roselle et al., 1998).

Green intrinsic fluorescence and red intrinsic fluorescence have also been reported in filamentous Gram-positive *Streptomyces* spp. (Willemse and van Wezel, 2009). These soil-dwelling bacteria exhibit a complex life cycle and are well-known for the plethora of natural compounds they produce, many of which are being used in human and veterinary therapies (Hopwood, 2007). With the exception of a very recent work by Meschke et al. (2012), red autofluorescence (RAF) has only been mentioned in the literature as noise background in studies aimed to monitor the dynamic localization of *Streptomyces* proteins fused to the monomeric red fluorescent protein (mRFP1) or its improved form mCherry (Nguyen et al., 2007; Willemse and van Wezel, 2009). Nevertheless, *Streptomyces coelicolor* and *Streptomyces lividans*, two close relative model strains that have now been used for decades for both physiological (metabolite production) and morphological (sporulation) differentiation studies, produce red-fluorescing secondary metabolites. These are the prodiginines (PdGs) or prodigiosin-like pigments, which are a family of tripyrrole red pigments that contain a common 4-methoxy-2,2′-bipyrrole ring system (Furstner, 2003) and exhibit maximum fluorescence at around 570 nm (Gerber and Lechevalier, 1976; Williamson et al., 2006). Next to the previously described antibacterial, antifungal, antiprotozoal, antihelmintic, antiviral, and antimalarial properties (Furstner, 2003), prodigiosin-like derivative drugs recently gained increasing interest due to their promising immunosuppressive and anticancer activities (Williamson et al., 2007). Alternatively, it is projected that prodigiosin-like pigments will be important natural dyes of the textile industry and substitute pigments to synthetic colorants for food, cosmetics, and pharmaceuticals due to their lower toxicity and reduced allergic reactions (Alihosseini et al., 2008; Siva et al., 2012; Venil and Lakshmanaperumalsamy, 2009). The chemical synthesis of PdGs is very complex and expensive. Important efforts are thus made for engineering PdG-producing microorganisms, and for uncovering novel nutrient substrate and culture conditions to generate higher production yields that are economically cost-effective (Giri et al., 2004; Siva et al., 2012; Stankovic et al., 2012).

In *S. coelicolor* PdG production is dependent on the *red* biosynthesis gene cluster (Rudd and Hopwood, 1980) in which expression is under the control of pathway-specific activators *redZ* (White and

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Bibb, 1997) and *redD* (Narva and Feitelson, 1990). The red pigmentation is a complex mixture of different PdGs where streptorubin B and undecylprodigiosin are the most dominant forms in *S. coelicolor* and in *S. lividans*, respectively (Meschke et al., 2012; Mo et al., 2008). Interestingly, PdG biosynthesis in *S. coelicolor* is growth-phase dependent (Hobbs et al., 1990; Huang et al., 2001; Takano et al., 1992). Its production coincides with the onset of the short period of growth cessation during exponential growth (i.e., the so-called transition phase), which marks the beginning of the switch to secondary metabolite production in liquid culture and is connected to the transition to the aerial life style (sporulation) on solid cultures (Granozzi et al., 1990; Puglia et al., 1995; Takano et al., 1992). PdG-associated fluorescence could thus be used as a natural marker of the physiological status of *S. coelicolor*.

Using *S. coelicolor* as a PdG-producing model bacterium, we show in this work how to monitor the red autofluorescence of prodigiosin-like pigments, and how this can be exploited by means of confocal microscopy in bacterial developmental studies.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. coelicolor M145 (Kieser et al., 2000) was used as parental strain, and strain M510 (Δ*redD* mutant) was used as PdG biosynthesis negative mutant (Floriano and Bibb, 1996). *S. coelicolor* strains were grown on the solid R2YE medium prepared as described previously (Kieser et al., 2000). Strains were grown on soya flour mannitol agar plates for spore stock preparations (Kieser et al., 2000) and 2.10^7 spores were used to inoculate agar plates.

2.2. Pigment extraction and sample preparation

Prodigiosin-like pigments were either methanol extracted or collected from crude extracts after mycelium sonication. *S. coelicolor* mycelia were scraped with a spatula from the agar plates covered with cellophane discs, put into 2 ml tubes and frozen at −70 °C. Twenty mg of frozen mycelia were subsequently incubated with 1 ml of acidified methanol (pH 2), vortexed and left shaking 15 min for pigment extraction. The suspensions were centrifuged at 14,000 rpm for 5 min, and, whenever required, additional extraction rounds were performed until mycelia were completely colorless. Volumes of different samples were then adjusted according to the sample that required the greatest number of extraction rounds (*S. coelicolor* M145 grown for 50 h on R2YE in this study). Alternatively, frozen mycelia were suspended into 500 µl of prodiginine extraction (PE) buffer (Tris–HCl 50 mM pH 7.5, $MgCl_2$ 5 mM, NaCl 50 mM, 1 mM DTT, SDS 1%, and glycerol 2%), sonicated for 10 min with 30 s pulses (Bioruptor, Diagenode, Liège, Belgium), centrifuged at 14,000 rpm for 15 min (repeated twice), and the PdG containing supernatants were used for absorbance and fluorescence analyses.

2.3. Colorimetric and fluorescence assays

One hundred µl of methanol extracts were deposited into wells of microtiter-plates (Nerbe Plus transparent flat shape and Greiner black flat shape for absorbance and fluorescence assays, respectively). Absorbance was measured at 530 nm, whereas the fluorescence emission was recorded at 570 nm, after excitation at 543 nm, using a Tecan *Infinite 200 PRO* Multimode Microplate Reader. Supplementary Fig. S1 shows the dilution series required to accurately analyze a non-saturated red fluorescent signal from *S. coelicolor* extracts. For the in-gel semi-quantitative analysis 50 µl of crude extracts in PE buffer were loaded into wells of a 1% agarose gel and, after electrophoresis, RAF images were taken with a *Typhoon* TRIO+ Fluorimager (excitation at 532 nm, 580BP30 emission filter). Semi-quantitative analysis of bands displaying RAF into the agarose gel was performed using the ImageJ software.

2.4. Prodiginine in situ visualization by confocal microscopy

Agar cubes of the R2YE medium inoculated with spores of *S. coelicolor* were extirpated out of the Petri dish and confluent lawns were sliced manually (transversal section) using a razor blade (the thickness of slices was approximately 0.2 mm) and sample slices were placed onto a 1 mm thick glass slide for direct microscopy analyses. Observations were made with a Leica TCS-SP2 confocal laser-scanning microscope. Samples were excited at a wavelength of 543 nm and emission was examined between 560 and 620 nm as described previously (Tenconi et al., 2012).

2.5. Image processing and 3D reconstruction of Streptomyces filament producing prodiginines

Z-Stacks of confocal images (objectif HCX PL APO 63 × 1.20 W CORR UV and pixels 512 × 512) derived from slice culture scanning were processed using the Fiji (ImageJ) software. For analyses of dense culture across a transversal section, ten Z-stack images from a 238.1 µm section were used for a standard deviation Z-projection (for light images) or a maximal Z-projection after applying a Gaussian Blur filter with a radius of 1 (for RAF images). For the 3D confocal image stack reconstitution we used the UCSF *Chimera* software to visualize 84 z-stack images from a 85.2 µm section (objectif HCX PL APO 63 × 1.20 W CORR UV with a zoom of 2.8 and pixels 512 × 512) preprocessed by the Fiji software (Gaussian Blur filter for RAF image stack).

3. Results

3.1. RAF of S. coelicolor is strictly associated with prodiginine biosynthesis

In order to validate the exploitation of the RAF of *S. coelicolor* to assess production levels of PdGs – between various strains and/or under different culture conditions – we first demonstrated that RAF was strictly associated with the activity of the *red* biosynthetic cluster and not related to other intracellular primary or secondary metabolites. PdG production was compared between *S. coelicolor* M145 (parental strain) and its Δ*redD* mutant (strain M510, impaired in PdG production (Floriano and Bibb, 1996)) grown for 50 h on R2YE, in the presence or absence of N-acetylglucosamine (GlcNAc) supply, which is well documented to block development under rich culture conditions and therefore limiting PdG production (Craig et al., 2012; Rigali et al., 2006; Rigali et al., 2008; Swiatek et al., 2012). The mycelium of *S. coelicolor* cultures was harvested from agar plates covered by cellophane discs and intracellular pigments were methanol extracted.

Fig. 1 shows the red pigmentation (REP) and autofluorescence from methanol extracts (panel A), and the semi-quantitative analysis of REP and RAF between the two different *S. coelicolor* strains, and under the two culture conditions tested (panel B). The measured REP and RAF were arbitrarily fixed to 100% for the parental strain M145 grown in R2YE agar plates. REP was not detectable in the Δ*redD* mutant, whereas RAF was estimated to about 2% of the amount produced by the parental strain M145 (Fig. 1A and B). When the parental strain M145 was grown for 50 h with GlcNAc, REP and RAF were reduced to about 20 (±1.5) and 18 (±0.3)%, respectively (Fig. 1A and B). That both REP and RAF decreased similarly upon GlcNAc supply and are both dependent of the presence of the pathway-specific activator RedD strongly support the hypothesis that the RAF of *S. coelicolor* is associated with the activity of the *red* biosynthetic cluster responsible for PdG production.