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Alpha-ketoglutarate protects *Streptomyces coelicolor* from visible light-induced phototoxicity

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ABSTRACT

It has been known that some *Streptomyces* species, including the model strain *Streptomyces coelicolor*, are vulnerable to visible light. Much evidence demonstrated that the phototoxicity induced by visible light is a consequence of the formation of intracellular reactive oxygen species (ROS), which are potentially harmful to cells. In this study, we found that α-ketoglutarate (α-KG) has a protective role against the phototoxicity in *S. coelicolor*. It could be because that α-KG can detoxify the ROS with the concomitant formation of succinate, which mediates the cells getting into anaerobiosis to produce more NADH and maintain intracellular redox homeostasis, a situation that was demonstrated by overexpressing *gdhA* in *S. coelicolor*. This finding, therefore, connects the central metabolites with the bacterial resistance against phototoxicity effect induced by visible light.

1. Introduction

It has been well known that low-power visible light can enhance bacterial viability [1], while high intensity visible light kills bacteria under aerobic conditions [2,3]. Reactive oxygen species (ROS) can be generated by visible light in living cells, and endogenous cellular photosensitizers such as porphyrins and flavins may be involved in this process [4–6]. The ROS amounts generated by visible light are likely to be positively correlated with the strength of light, *i.e.* low-power visible light might induce low amounts of ROS, while high intensity visible light could generate high amounts of ROS. The ROS at low level participate in cell signaling processes, while excessive ROS result in oxidative stress at which cells could be damaged or killed [7].

Streptomyces is a genus of gram-positive bacteria renowned for its ability to produce a variety of antibiotics and other bioactive natural products [8]. Previous research has shown that light remarkably inhibited the spore germination of some *Streptomyces* species, including *Streptomyces viridosporus* and *Streptomyces coelicolor* [9]. The intracellular superoxide dismutase levels were found to be remarkably enhanced by the light under aerobic condition, indicating that light and oxygen together might produce high amounts of ROS. Most *Streptomyces* produce carotenoids under light induction [10]. Although carotenoids were known to protect cells from photodynamic damage in *Rhodobacter* species [11], these pigments were shown to have no protective role against the lethal effects of light in *Streptomyces* species [9]. And till now, little is known about how *Streptomyces* copes with the photo-oxidative stress.

α-ketoglutarate (α-KG), an important intermediate of the tricarboxylic acid (TCA) cycle, lies at the intersection between the carbon and nitrogen metabolic pathways, and acts as the major carbon skeleton for nitrogen-assimilating reactions [12]. Meanwhile, α-KG also acts as a regulatory molecule, and the number of metabolic pathways known to be regulated by α-KG has increased significantly in recent years [13]. Fedotcheva et al. found that α-KG can detoxify H_2O_2 through spontaneous decarboxylation to yield succinate [14]. Maillous et al. demonstrated that TCA cycle can be modulated under oxidative stress, and by which, α-KG production is increased for effectively diminishing ROS with concomitant curtailing the formation of NADH, a situation that further impedes the release of ROS [15]. The direct involvement of α-KG in resisting phototoxicity has not been conclusively demonstrated.

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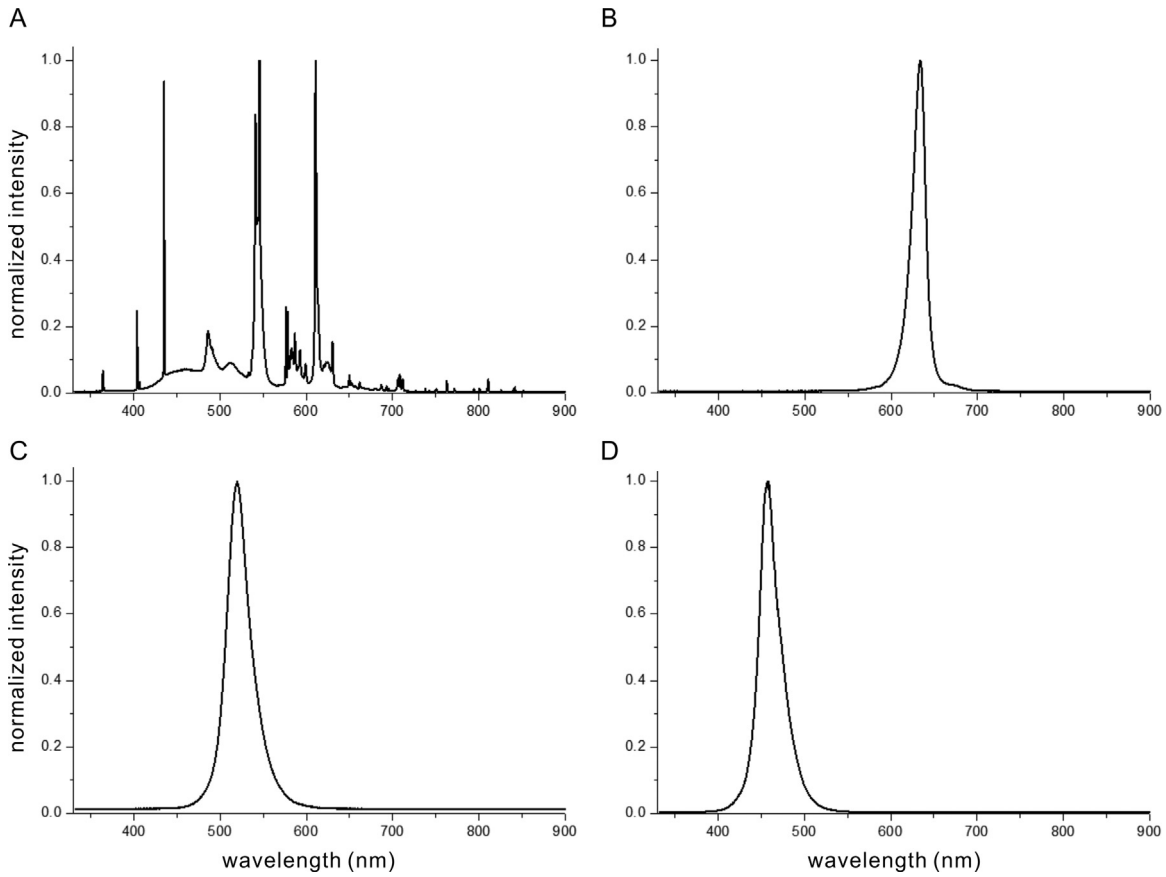

Fig. 1. Emission spectra of used light sources. (A) visible (white) light. (B) red light. (C) green light. (D) blue light.

Therefore, the purpose of the present study was to clearly show the protective role of α-KG against phototoxicity in S. coelicolor and further discuss the in-depth mechanism.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

Escherichia coli strain DH5α was used as the general cloning host. *E. coli* ET12567 [pUZ8002] was used as the donor in the intergenic conjugations. *E. coli* ET12567 is a methylation-defective strain (*dam-13*::Tn9, *dcm-6*, *hsdM*). pUZ8002 is a nontransmissible oriT-mobilizing plasmid [16]. *S. coelicolor* A3(2) M145 was used as a parental strain and designated the wild type.

E. coli strains were grown in Luria-Bertani (LB) broth and agar supplemented with antibiotics. Apramycin (50 μg/mL), chloramphenicol (25 μg/mL), kanamycin (50 μg/mL) were added to the growth media as required. Unless otherwise noted, *S. coelicolor* was grown on mannitol soya (MS) flour medium (20 g agar, 20 g mannitol, 20 g soya flour per 1 L water). Apramycin (50 μg/mL) was added to the growth media as required.

2.2. Light illumination

The effect of light on *S. coelicolor* was studied by plating 10 μl diluted spore-suspensions (about 3.5×10^8 spores) on MS agar supplemented if required with filter-sterilized α-KG, glutamate, ammonium or nitrate. Plates were incubated at 30 °C, 2 cm away from 13 W fluorescence lamp (YPZ220/13-2U, OPPLE). Color temperature is 6500 K for this light source. Illuminance level was 9000 lx on the plates. Red (623–640 nm), green (506–537 nm) or blue (446–473 nm) LED lamps (5 W, CQ-LV8003C, OEM) were also used for illumination experiments. The corresponding illuminances were 8500 lx, 6700 lx and 950 lx on plates, respectively. Light spectra and illuminances of these light sources were measured by using AvaSpec-Mini 3648 spectrometer (AVANTES, Netherlands) and TES 1332 A Digital Lux Meter (TES, Taiwan), seperately. Half of the upper surface of plates was covered with opaque papers, while the other half was transparent, which was indicated in the Fig. 2A. The bacterial cells were cultured for 6 days and then images were taken for phenotypic analysis. The phenotypic analyses were quantified by counting *S. coelicolor* spore colony-forming unit (CFU) at each plate, which were performed by serial diluting these spores on LB agar. Three independent experiments were repeated for each phenotypic analysis, and each time experiment used independent samples.

2.3. Overexpression of gdhA in S. coelicolor

The emrE promoter region was PCR amplified with the high-fidelity Dpx DNA polymerase (Tolo Biotech., Shanghai, China), using primers of emrEP-F (5′-ggtaccagcccgacccgagc-3′) and emrEP-R (5′-cgctggatcctaccaaccggca-3′). The coding DNA sequence of *gdhA* in *S. coelicolor* was PCR amplified with primer pairs of gdhA-F (5′-aaaacaagcttcacggaggtacggacatggtgcccgccgtgccagaaag-3′) and gdhA-R (5′-aaaagtctagaaagagcgcttccgacggcac-3′). Two amplicons were digested with *Hind*III and *Xba*I, ligated and was then cloned into the *HindIII* and *Xba*I sites of the integrative plasmid of pSET152, obtaining pSET*gdhA*, in which the *gdhA* structure gene was fusioned with the *emrE* promoter. Plasmids pSET*gdhA* and its control pSET152 were firstly transformed into *E. coli* ET12567 [pUZ8002] and then conjugated to M145 for further illumination tests.

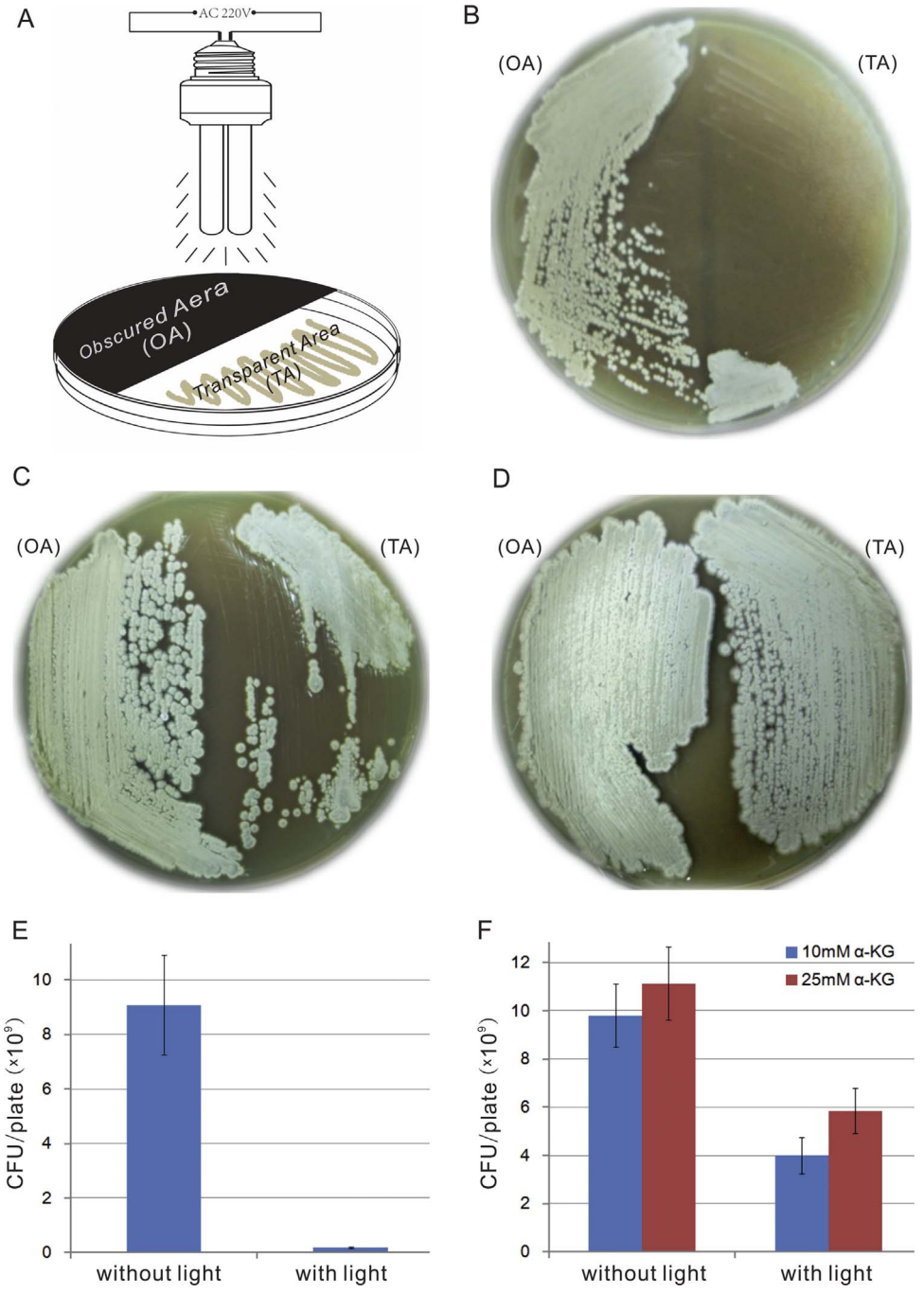

Fig. 2. *S. coelicolor* is sensitive to visible light illumination and α-KG protects *S. coelicolor* from it. (A) Schematic diagram of light illumination. Obscured Area (OA) was covered with opaque paper; Transparent Area (TA) indicates the region of plate without any covering. (B) *S. coelicolor* was cultured on MS medium. Visible light phototoxicity against *S. coelicolor* was indicated from the observation that the number of bacterial cells on TA is far less than that on OA. (C & D) *S. coelicolor* was grown on MS plate supplemented with either 10 mM (C) or 25 mM (D) α-KG. (E & F) Viable spores were estimated by counting CFU at each plate for *S. coelicolor* cultured on above conditions, respectively. Compared Fig. 2F with E, results indicated that α-KG can apparently decrease the visible light phototoxicity. n=3, mean ± S.D., $p < 0.05$.

2.4. Effect of light on spores germinating of S. coelicolor

We inocubated Petri dishes containing growth medium with 10 µl appropriately diluted spores of *S. coelicolor* (about 3.5×10^8 spores). A separate lot of Petri dishes were first incubated in the dark for variable periods (0 h, 6 h, 12 h, 24 h and 36 h) and then in the light. The total culture time length was 6 days for each Petri dish. The same fluorescence lamps and illumination conditions as above stated were used in this analysis.

2.5. Measurement of intracellular α-KG

Levels of α-KG were determined using the Alpha-Ketoglutarate Assay Kit (BioVision Inc., Milpitas, CA, USA) following instructions provided by the manufacturer. Bacteria cultured 4 days were collected from glassine paper covered on solid medium, and suspended with α-KG Assay Buffer (5 µl/mg wet weight). A sterile 5 mm steel bead (Qiagen, Valencia, CA) and 100 µl sterile 0.1 mm glass beads (Scientific Industries, Inc., NY, USA) were added for complete bacterial lyses in a TissueLyser II (Qiagen, Valencia, CA), run at 30 Hz for 10 min cell suspension were heated to 95 °C for 5 min followed centrifuging. The

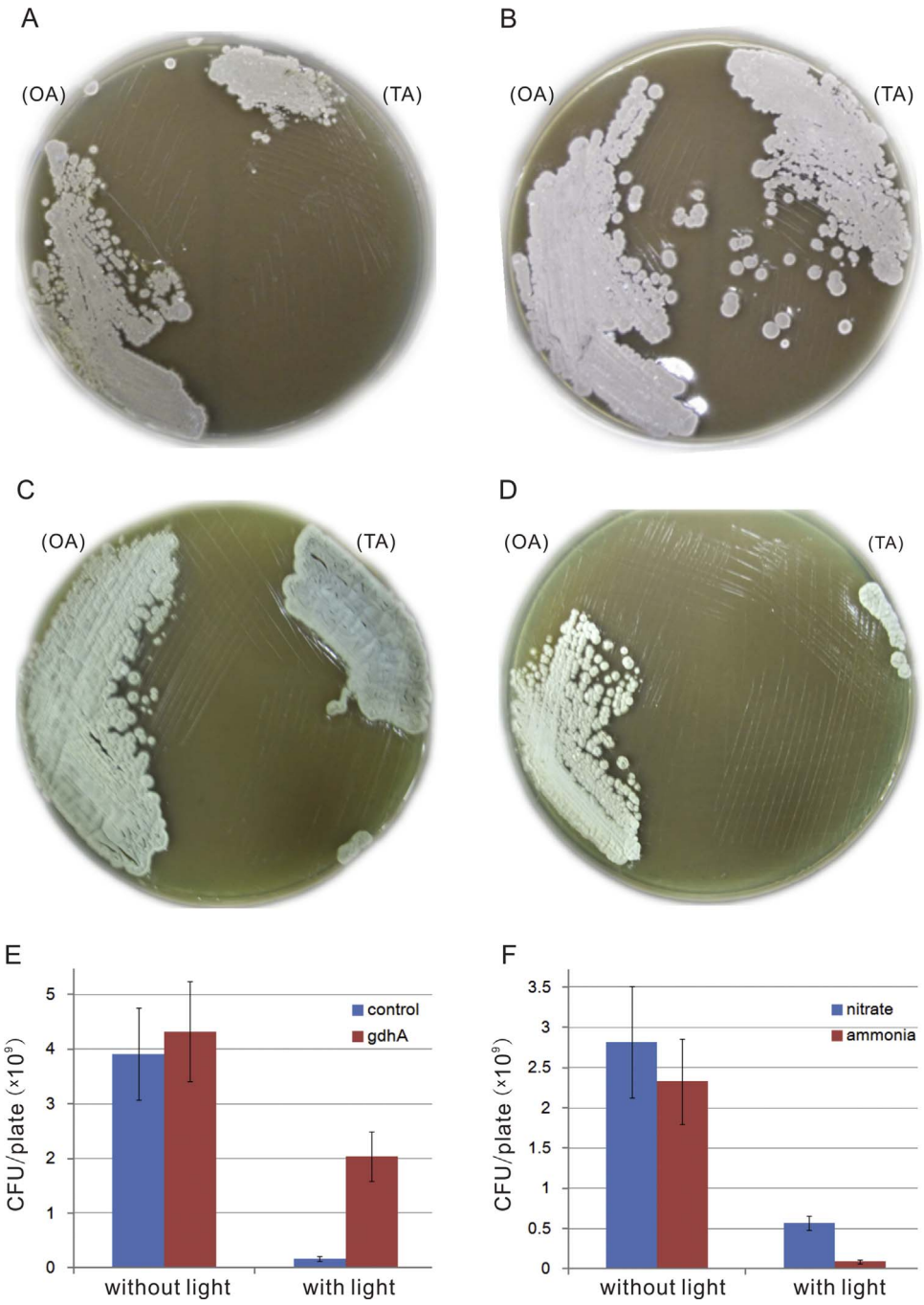

Fig. 3. The influence of overexpressed gdhA and different nitrogen sources on the growth of *S. coelicolor* under light illumination. (A & B) *S. coelicolor* spores harboring either the control plasmid pSET152 (A) or the plasmid overexpressing *gdhA* (B) were grown on MS plate. (C & D) *S. coelicolor* was cultured on MS medium supplemented with either 25 mM nitrate (C) or 100 mM ammonia (D). (E & F) Spores CFU counting on each plate were performed with above growth conditions, respectively. They were compared with Fig. 2E, and results demonstrated that both overexpressed *gdhA* and nitrate protected the strain while ammonia had no protective role against light illumination. n=3, mean ± S.D., $p < 0.05$.

extracted samples were further deproteinized by passing through a 10-kD cut-off spin column. The concentration of α-KG was quantified by reading fluorescence value using Ex/Em=535/587 nm.

2.6. NAD/NADH ratio measurement

NAD/NADH ratio was determined by using NAD/NADH Quantification Kit (Sigma-Aldrich, St. Louis, MO, USA). The same samples as used for α-KG measurement were suspended with NAD/NADH Extraction Buffer (5 µl/mg wet weight). Cells were rapidly lysed by using TissueLyser II (Qiagen, Valencia, CA) as above. Samples were centrifuged and the supernatant was transferred into a 10 kDa molecular weight cut off spin column for deproteinizing sample. 200 µl of the extracted samples were heated to 60 °C for 30 min in a heating block to decompose NAD^+. NADH was quantified by measuring the absorbance at 450 nm. Background was corrected by subtracting the blank value from all readings. The ratio of NAD/NADH in a sample was determined as: $[NAD_{total} - NADH]/NADH$.

2.7. Statistitical analysis

Statistical analysis was performed as indicated in the figure legends

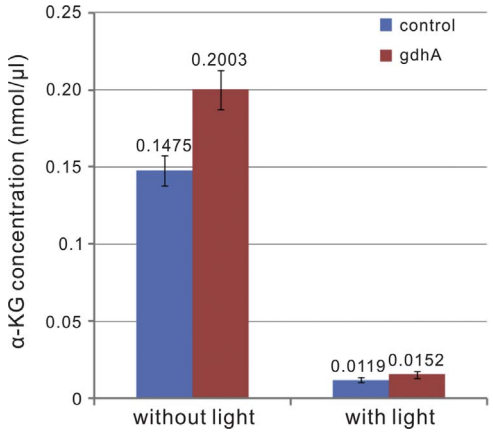

Fig. 4. Measurement of the *in vivo* α-KG concentration in *S. coelicolor*. Intracellular α-KG concentration in illuminated strains were much lower than that in strains without light illumination. The α-KG concentration in *gdhA* overexpressed strains was slightly higher than that in the control strains both in the light (36% higher) or dark (27% higher). n=3, mean ± S.D., $p < 0.05$.

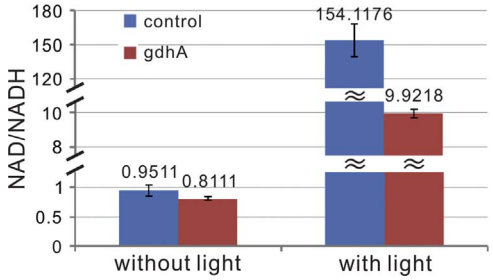

Fig. 5. Measurement of the *in vivo* NAD/NADH ratio of *S. coelocolor* strains with or without light illumination. Intracellular NAD/NADH in illuminated strains was much higher than that in strains cultured in dark. The NAD/NADH concentration in *gdhA* overexpressed strains was lower 17% than that in the control strains when both of them were cultured in dark. When both of them were cultured in light, the NAD/NADH ratio in the *gdhA* overexpressed strain was much lower than that in the control strain. n=3, mean ± S.D., $p < 0.05$.

using the standard statistical software IBM SPSS Statistics version 23 for Windows. Statistical significance was assessed by the Student's *t*-test, and two-tailed *p* values of less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. S. coelicolor *is sensitive to visible light illumination*

Spores of *S. coelicolor* were spread on growth medium and incubated 6 days either in the light or dark (Fig. 2**A**). The emission spectrum of the visible light is shown in Fig. 1A. The growth results showed that *S. coelicolor* was sensitive to visible light, and spores could barely grow at transparent area under light illumination, in contrast to the abundant spores growing at obscured area (Fig. 2**B and E**). Therefore, similar to the previous discovery revealed by Imbert et al. [9], visible light has a phototoxic impact against the growth of *S. coelicolor*.

S. coelicolor was then individually illuminated with light of different wavelengths, *i.e.* red light, green light and blue light, and was found to be more sensitive to the blue light illumination (Fig. S1), which was the same as those reported in other microbes [2,17]. The emission spectra of these three light sources are shown in Fig. 1B, C and D, respectively. The endogenous cellular photosensitizers can absorb the entire range of visible light, but with a maximum in the blue region; meanwhile, ROS production was found to be the highest under blue light illumination [17], which might well explain the highest phototoxicity produced by blue light. In addition, silimar to those reported in *S. viridosporus* [9], *S. coelicolor* was more sensitive to visible light illumination in the first several hours since plating (Fig. S2), which corresponds to the early stage of germination, including germ tube emergence, vegetative mycelium growth and the first round of programmed cell death (PCD) [18]. As described by Beites *et al.*, an excessive oxidative stress inhibited the first round of PCD and then blocked aerial mycelium formation in *S. natalensis* [19], visible light probably produces oxidative stress that inhibits the early germination of S. coelicolor. Macleanet al. found that inactivation of bacterial endospores or fungal dormant spores needs higher energy doses than that of vegetative cells or germinating spores when they are irradiated by 405 nm visible light [20,21]. So it is possible that dormant spores or mycelia of S. coelicolor can be inactivated by visible light if the energy dose of irradiation is high enough. Germinating spores of S. coelicolor might be most sensitive to visible light phototoxicity.

3.2. *α-KG protects* S. coelicolor *from visible light induced phototoxicity*

As intermediates such as α-KG could diminish ROS, α-KG at different concentrations (*i.e.* 10 mM and 25 mM) was tested for its role in protection of *S. coelicolor* against light illumination, with the finding that α-KG concentration had a positive relation with the protective effect (Fig. 2**C, D and F**). Although α-KG can be mutually converted to glutamate *in vivo*, supplementation of glutamate had no role in protecting *S. coelicolor* against phototoxicity (Fig. S3). To further demonstrate the protective role of α-KG, the NADP-dependent glutamate dehydrogenase encoding gene, *gdhA*, was overexpressed in *S. coelicolor*, through employing a constitutive expressing *emrE* promoter. GdhA catalyzes reversible reactions, *i.e.* either the reductive amination of α-KG to yield glutamate or the oxidative deamination of glutamate to produce α-KG [22]. It was found that overexpression of *gdhA* greatly protected the spores from phototoxicity (Fig. 3**A, B and E**). In the meantime, the concentration of intracellular α-KG was measured, and it was found that the α-KG concentration was slightly higher in *gdhA* overexpressed strain than in the control strain both in the light (36% higher) or dark (27% higher) (Fig. 4), indicating that GdhA mainly catalyzes the oxidative deamination process to produce α-KG. Therefore, the protective role by overexpression of *gdhA* was consistent with the observed results of α-KG supplementation. In *S. coelicolor*, there is the other glutamate dehydrogenase GdhB, which catalyzes similar reaction as GdhA [23]. However, the GdhB is an NAD(H)-specific enzyme, which can catalyze generation of α-KG, meanwhile NADH is also produced. This reaction might be unfavorable because of increased NADH promoting ROS formation under oxidative stress [15]. For this reason, we did not use gene *gdhB* for this experiment. In addition, intracellular α-KG concentration in illuminated strains was found to be much lower than that in strains on obscured area (Fig. 4). Although Alhasawiet al. found that α-KG might be involved in transamination reaction with glycine to form glyoxylate for combating oxidative stress in Pseudomonas fluorescens [24], more researches demonstrated that α-KG was depleted for detoxifying ROS to form succinate in oxidative stress [14,15]. Thus, intracellular α-KG might be depleted to oppose ROS under light illumination.

As α-KG is a key intermediate for nitrogen assimilation, supplementation of different nitrogen sources may also alter the intracellular α-KG concentration. For example, addition of ammonia may quickly consume a large amount of intracellular α-KG to produce glutamate, which would sharply reduce the intracellular concentration of α-KG. While with the supplementation of nitrate, which is mainly assimilated by the glutamine synthetase with the consumption of glutamate, the intracellular α-KG will not be greatly influenced [25,26]. Moreover, because nitrate is an unfavorable nitrogen source for bacteria, addition of nitrate probably reduce the nitrogen assimilation rate and may in turn protects the intracellular α-KG pool, therefore providing protec-

tive effects [25]. Based on this hypothesis, *S. coelicolor* spores were cultured on medium with either 25 mM nitrate or 100 mM ammonium, with the finding that nitrate protected the strain to some extent from visible light-induced phototoxicity while ammonia did not obviously have this protective role (Fig. 3**C, D and F**).

3.3. α-KG maintains NAD/NADH redox homeostasis in S. coelicolor

It is well known that NAD/NADH ratio is an index of cellular reducing potential [27]. The NAD/NADH ratio in both *gdhA* overexpressed strain and the control strain was measured using same samples as above intracellular α-KG measurement, which includes both irradiated and non-irradiated bacterial cells (Fig. 5). The results showed that the NAD/NADH ratio kept at a relatively low level in both strains when cultured in dark, and the ratio was 17% lower in the *gdhA* overexpressed strain than that in the control strain. As shown in Fig. 4, the *gdhA* overexpressed strain produced more α-KG than the control strain without light illumination. The increased α-KG promoted the TCA cycle, which formed more NADH, and consequently NAD/NADH was decreased. However, under the light illumination condition, the intracellular NAD/NADH ratio in both strains increased drastically, but the ratio in the *gdhA* overexpressed strain was much lower (*i.e.* 15 folds) than that in the control strain. Aerobic respiration relies on O_2 to drive ATP production. This process is also accompanied by the formation of ROS, and this situation can be exacerbated when NADH is abundant under oxidative stress [28]. Hence an oxidatively stressed organism will strive to decrease NADH production by reconfiguring its metabolic processes in order to limit ROS formation [29]. For instance, *P. fluorescens* decreased the production of NADH by using diverse strategies including modulating TCA cycle, increasing NADK activity and improving NADH to NADPH conversion cycle during oxidative stress [15,30,31]. Therefore, we can reasonably deduce that *S. coelicolor* also decreased its NADH formation so that its NAD/NADH ratio is increased during photo-oxidative stress (Fig. 5).

As stated above, the *gdhA* overexpressed strain produced more α-KG than the control strain. A lot of research have demonstrated that α-KG detoxified ROS to produce succinate, which may act as an intracellular mediator of anaerobiosis [32,33]. To date, the knowledge about how succinate promotes anaerobiosis in obligate aerobes is limited. In *Mycobacterium tuberculosis*, which belongs to the same phylum *Actinobacteria* as *S. coelicolor*, anaerobic adaptation was coupled to succinate accumulation and secretion [34]. Succinate secretion might help maintain membrane potential by H^+/succinate symport, which could drive the synthesis of ATP [33]. Succinate might also act as a signal molecule and help the cell activate its energy production *via* anaerobiosis with the aid of hypoxia inducible factor [32,35]. Global transcriptomic analysis of oxidatively stressed *Escherichia coli* found that aerobic respiration-related genes were downregulated and anaerobic genes were upregulated, which suggested a switch to anaerobic metabolism [36]. Although *S. coelicolor* can not grow in the absence of oxygen, its genome sequence reveals several enzymes that are associated with anaerobic respiratory metabolism [8]. It is capable of microaerobic growth and maintaining viability through several weeks of anaerobiosis [37]. Thus, the *gdhA* overexpressed strain might have more α-KG transformed into succinate which made the cells to produce more energy and NADH *via* anaerobiosis in order to survive under the photo-oxidative stress [38]. As a result, the *gdhA* overexpressed strain has lower NAD/NADH ratio than the control strain under light illumination (Fig. 5).

In sum, the *gdhA* overexpression produced more α-KG in *S. coelicolor* under visible light illumination, and consequently α-KG detoxified ROS to form succinate, which mediate the cells getting into anaerobiosis. By this way, the *gdhA* overexpressed strain produced more energy and NADH and maintained redox homeostasis to survive in photo-oxidative stress. This is a possible mechanism by which α-KG protects *S. coelicolor* from phototoxicity and extends cellular longevity.

Acknowledgement

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Appendix A. Supplementary material

Transparency document associated with this article can be found in the online version at doi:http://dx.doi.org/10.1016/j.bbrep.2016.11.002.

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