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Generation of reactive oxygen species upon red light exposure of cyanobacteria from Roman hypogea

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ABSTRACT

Subterranean archaeological sites in Rome (Italy) are threatened by phototrophic biofilms predominated by cyanobacteria and associated microorganisms. They damage the frescoes, mortar, marble, and tufa rock wherever artificial lighting is installed. During the past two decades, the conservation strategies have evolved gradually; rather than restricting the illumination time and intensity, the latest approach is to use strong light to reduce their growth. Since cyanobacterial cells are abundant in phycobilisomes and chlorophyll *a*, which produce reactive oxygen species (ROS) upon irradiation, strong red light (620–650 nm) was applied to generate high amount of ROS in a rate beyond the quenching capacity of the organism. After 25 h of irradiation, the photosystem II quantum yields of seven cyanobacterial isolates in culture were reduced by 65–94%. Conversely, blue light (460–480 nm) promoted photosystem II activity by up to 35%. δ-Aminolevulinic acid (D-ALA) was introduced to enhance the treatment, as it can be transformed into protochlorophyllide by cyanobacteria and then excited by red light to generate ROS inside the cells. Since the natural photosynthetic pigments as well as the endogenous protochlorophyllide exist only within the cyanobacterial cells, they are unlikely to contaminate or damage the underlying stone substrata. Electron spin resonance spectroscopy confirmed that D-ALA treatment caused the formation of ROS; spin trap experiments indicated that radicals were produced in the system.

1. Introduction

Catacombs and other subterranean archaeological sites in Rome (Italy) are extensive underground sites built by ancient Romans and Christians tens of centuries ago. Because of their great archaeological and historical importance, artificial lighting has been installed in these sites to allow access to tourists and researchers from all over the world. However, the presence of light and the constant temperature (16.8–18.0 °C), high CO_2 concentration, and high relative humidity (80–99% RH) all year round serve as an incubator for photoautotrophic organisms (Albertano, 2012). The pioneer microbes on the stone substrata are mostly cyanobacteria and a few eukaryotic microalgae, whose colonization provides an excellent organic nutrient base for subsequent heterotrophic microbiota and for their biodeterioration activities (Albertano and Urzì, 1999; Hernández-Mariné et al., 2003; Crispim and Gaylarde, 2004; Zammit et al., 2011a,b). Hence, the inhibition of cyanobacterial growth is the ultimate approach to rid subterranean archaeological sites of microbial attack (Albertano et al., 2003, 2005; Urzì et al., 2010).

Photodynamic antimicrobial chemotherapy (PDT) is a novel method to suppress bacterial growth. It involves the interaction of light with photosensitizers, which are compounds with highly conjugated double bonds where electrons can be easily promoted to the excited state by specific wavelengths of light. The excited photosensitizers can either exchange electrons or protons with adjacent molecules to generate superoxide radicals, hydroxyl radicals, and hydrogen peroxide (Type I reaction); or directly transfer energy to oxygen molecules, thereby producing singlet oxygen (Type II reaction). All these reactive oxygen species (ROS) are highly oxidative and toxic to living cells. The obvious merit is that the bacteria can hardly become resistant to ROS (Dennis et al., 2003).

Despite the remarkable effect of PDT against bacteria (Szocs et al., 1999; Bertoloni et al., 2000), fungi (Friedberg et al., 2001), and viruses (Bockstahler et al., 1979; O'Brien et al., 1992), very few studies have focused on cyanobacteria. Drábková et al. (2007) and

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Jančula et al. (2008) have tested the algicidal effects of 31 phthalocyanine compounds against the cyanobacterium *Synechococcus nidulans* from eutrophic and hypertrophic waters, and McCullagh and Robertson (2006) and Young et al. (2008) in the EU project BIODAM have examined the potential of methylene blue and nuclear fast red to reduce cyanobacterial growths on stone monuments. However, many phthalocyanine compounds are water-insoluble, and even worse, all these photosensitizers have intense intrinsic colors that will noticeably stain the surfaces of historic cultural properties. Even though hydrogen peroxide was reported to be able to break down the residual photosensitizer on the monuments (McCullagh and Robertson, 2006; Young et al., 2008), the strong oxidation potential of hydrogen peroxide (1.78 V) will inevitably bleach and damage the valuable substrata. In addition to the oxidative damage, the end products of complete oxidative mineralization of methylene blue ($C_{16}H_{18}N_3SCl$) are some strong acids, including nitric acid, sulfuric acid, and hydrogen chloride, as indicated in the stoichiometric equation (Mills et al., 1993; Sopajaree et al., 1999; Salem and El-Maazawi, 2000):

$$C_{16}H_{18}N_3SCl_{(s)} + 52H_2O_2 \rightarrow 16CO_{2(g)} + 0.5O_{2(g)} + 58H_2O + 3HNO_3 + H_2SO_4 + HCl$$

Therefore, the search for a safe and effective photosensitizer is necessary to avoid the staining and oxidative damage to the stone substrata. One of the most promising candidates is the endogenous protochlorophyllide precursor δ-aminolevulinic acid (D-ALA), which is a colorless compound that is mild to the substrata and can easily be neutralized just before use. Hence, this study was planned to test the effectiveness of D-ALA-mediated PDT against cyanobacterial isolates and biofilms collected from hypogea and catacombs in Rome (Italy), and to compare the usefulness of different wavelengths of monochromatic light to inhibit cyanobacterial growth.

2. Materials and methods

2.1. Cyanobacterial sampling

Phototrophic biofilms were carefully peeled off the calcareous substrata in the Christian catacombs of St. Domitilla and St. Callistus along the Via Appia Antica in Rome in November, 2011, by using the non-invasive adhesive tape method in order to minimize any damage to the valuable substrata or to the integrity of biofilm structure (Urzì and De Leo, 2001).

Seven cyanobacterial strains previously isolated from phototrophic biofilms in five hypogea and catacombs in Rome were also used (Table 1) (Bruno et al., 2009). They were cultivated in BG11 or BG11o medium (Rippka et al., 1979) at 20 ± 1 °C, 60% RH under white fluorescent light (1.2 μmol photons m^{-2} s^{-1}) in a dark/light cycle of 12 h/12 h.

Table 1
List of cyanobacterial strains used in the experiments.

Strain number	Species	Order	Medium	Sampling site
CP5	*Eucapsis* sp.	Chroococcales	BG11	Catacombs of Priscilla
CP6	*Scytonema julianum*	Nostocales	BG11o	Catacombs of Priscilla
VRUC135	*Oculatella subterranea*	Oscillatoriales	BG11	Domus aurea
VRUC136	*Leptolyngbya* sp. (green)	Oscillatoriales	BG11	Domus aurea
VRUC206	*Leptolyngbya* sp. (green)	Oscillatoriales	BG11	Domus aurea
CSC7-21	*Scytonema julianum*	Nostocales	BG11o	Catacombs of St. Callistus
NAV10bis	*Symphyonemopsis* sp.	Stigonematales	BG11o	Roman necropolis, Vatican carparking

2.2. PDT experiments

The PDT experiments were conducted inside specially designed illumination cabinets (M2M Engineering, Italy). The irradiances were provided by LED lamps with different ranges of wavelengths (Osram, China) peaked respectively at red 650 nm, red 630 nm, orange 600 nm, green 535 nm, green 520 nm, blue 480 nm, and blue 460 nm, or producing white light (Fig. 1).

The photosensitizer, δ-aminolevulinic acid (D-ALA), was purchased from Sigma–Aldrich (Milan, Italy). D-ALA was dissolved in ddH_2O and neutralized with $NaOH_{(aq)}$ just before every use.

Phototrophic biofilms and cyanobacterial isolates from catacombs were incubated with 30 mM D-ALA in the dark overnight. They were irradiated under the monochromatic LEDs, and the quantum yield of photosystem II was regularly measured using a pulse-amplitude-modulated chlorophyll fluorometer (mini-PAM) (Walz, Germany) during LED light exposure.

2.3. Detection of radicals

Cyanobacterial isolates were rinsed three times with 6 mM EDTA to remove trace metallic cations in the medium. The cultures were incubated with various concentrations of D-ALA in the dark overnight. The cells were then either sonicated on ice for 20 min or broken with liquid nitrogen under dim light, and 40 mM N-tert-butyl-α-(4-nitrophenyl)-nitrone was added as spin trap. Approximately 50 μl of samples was drawn into glass capillaries and sealed. The samples were irradiated continuously with red light (625 nm, 50 μmol photons m^{-2} s^{-1} photosynthetically active radiation) during ESR measurement. The ESR spectra were measured at room temperature with a Bruker ESP300 X-band spectrometer equipped with a TE_{110}-mode resonator, using 20 mW power at 9.81 GHz, and the spectra were recorded using 2.63 G modulation and 100 G scanning in 21 s. A total of 16 spectra were accumulated in 6 min to improve the signal-to-noise ratio.

2.4. Confocal laser scanning microscopy (CLSM) imaging

A CLSM (Olympus PUS FV1000) was employed to observe the viability of cells in the biofilm during the PDT process. Biofilm images in *x*–*y*, *x*–*z*, and *y*–*z* dimensions were acquired in maximum intensity projection mode. Biofilm damage was visualized with SytoX Green® staining (Invitrogen) and multi-channel laser scanning microscopy techniques (Billi, 2009). The 3-D rendering images were processed with Imaris 6.2 software (Bitplane AG Zurich, Switzerland).

3. Results

3.1. Effect of monochromatic light on the viability of cyanobacterial isolates

The seven cyanobacterial isolates tested altered their PSII quantum yield drastically in response to the different intensities and wavelengths of monochromatic light.

Even moderate light intensities (approx. 15 μmol photons m^{-2} s^{-1}) generally reduced cyanobacterial viability. The PSII quantum yields were reduced by 64–94% in all the seven isolates after 25 h irradiation (Figs. 2–8). Noticeably, the same LEDs with lower irradiance (approx. 1.4 μmol photons m^{-2} s^{-1}) had