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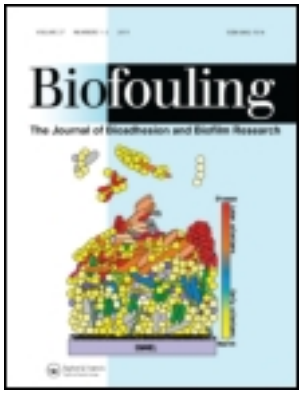

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Characteristics and role of the exocellular polysaccharides produced by five cyanobacteria isolated from phototrophic biofilms growing on stone monuments

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Characteristics and role of the exocellular polysaccharides produced by five cyanobacteria isolated from phototrophic biofilms growing on stone monuments

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Three coccoid and two filamentous cyanobacterial strains were isolated from phototrophic biofilms exposed to intense solar radiation on lithic surfaces of the Parasurameswar Temple and Khandagiri caves, located in Orissa State, India. Based on to their morphological features, the three coccoid strains were assigned to the genera *Gloeocapsosis* and *Gloeocapsa*, while the two filamentous strains were assigned to the genera *Leptolyngbya* and *Plectonema*. Eleven to 12 neutral and acidic sugars were detected in the slime secreted by the five strains. The secretions showed a high affinity for bivalent metal cations, suggesting their ability to actively contribute to weakening the mineral substrata. The secretion of protective pigments in the polysaccharide layers, namely mycosporine amino acid-like substances (MAAs) and scytonemins, under exposure to UV radiation showed how the acclimation response contributes to the persistence of cyanobacteria on exposed lithoid surfaces in tropical areas.

Keywords: phototrophic biofilms; cyanobacteria; sandstone biodeterioration; exopolysaccharides; UV-protective pigments; metal affinity; ETR (electron transport rate)

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Introduction

Biofilms growing on lithic surfaces of monuments have been intensively studied over recent decades since there is growing concern for the preservation of cultural heritage (de la Torre et al. 1993; Gaylarde and Morton 1999; Ramirez et al. 2010). Biological activity, which has been estimated as the cause of 20–30% of the deterioration of monuments (Wakefield and Jones 1998), leads to the staining of the lithic surfaces, the formation of mineral crusts as well as a form of decay called *patina*. Deterioration is the result of chemical and physical interactions between the biofilm and the substratum (Warscheid 1996). According to some authors, phototrophs (ie cyanobacteria and microalgae) are the first colonizers of stones (Lamenti et al. 2000; Crispim and Gaylarde 2005), being in many cases visible as dark pigmentation due to the presence of chlorophylls and carotenoids (Tiano et al. 1995; Sanmartin et al. 2010). However, epilithic biofilm-forming cyanobacteria discolour natural rocks, buildings and monuments on which they grow not only because of their chlorophyll and carotenoids, but in most cases also because of the production of phycobiliproteins and UV-screening compounds, such as the yellow-brown scytonemins and the red to blue gloeocapsins. The latter compounds are secreted in the EPS layers and are responsible for the dark pigmentation of stones (Albertano Forthcoming 2011).

Furthermore, developing photosynthetic biomass releases carbohydrates, growth factors and other nutrients, which can be used as substrates by chemoorganotrophic bacteria that may produce corrosive organic acids or oxidize metal cations, thereby causing the decay of the stones and enhancing biodeterioration (Donlan 2002; Urzì 2004).

The synthesis and excretion of extracellular polymeric substances (EPS), mainly produced by the cyanobacteria, is key to the spread of phototrophic biofilms, but EPS also enhances the deterioration of stone. EPS constitutes 50–90% of the organic carbon of the biofilm, being primarily composed of polysaccharides (Nielsen et al. 1997; Pereira et al. 2009). EPS provides a matrix that confers stability and cohesion to the biofilm along with protection against desiccation, high solar radiation and attack by protozoans. An effective shield against high energy radiation is provided by the synthesis of ultraviolet (UV) screening compounds mainly mycosporine amino acids (MAAs) and scytonemins secreted in the EPS layers (De Philippis and Vincenzini 2003; Pattainak et al. 2007).

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The presence of these slimy layers is essential for the protection and survival of the biofilm, but it presents a threat for the preservation of the lithic surfaces of monuments, which are subjected to mechanical stress (Reddy et al. 1996) and chemical interactions (Crispim and Gaylarde 2005) causing changes in their structure and visible darkening.

The work presented here was aimed at understanding the possible role of five exopolysaccharide-producing cyanobacterial strains in the biodeterioration of the lithic faces of two Indian stone monuments, in particular to determine the contribution of polysaccharides to the enhancement of biofilm resistance to environmental stress. The cyanobacteria were isolated from phototrophic biofilms exposed to intense solar radiation on the lithic surfaces of Parasurameswar Temple and Khandagiri caves, located in the state of Orissa, India.

Material and methods

Isolation of strains

Five cyanobacterial strains were isolated from biofilms growing on stone monuments located in Bhubaneswar, state of Orissa, in the eastern region of India. Biofilm samples were collected on April 2007 from the temple of Parasurameswar and the Khandagiri cave complex (20° 20′6.4″N, 85°44′48″E). The small temple of Parasurameswar is believed to be a good example of early Orissan architecture of the post-Buddhist period, dating as far back as *ca* 750 AD. Khandagiri caves are partly natural and partly artificial limestone caves situated on Khandagiri hill and are exposed to solar irradiance. The samples for this study were taken from rocks not directly exposed to solar irradiance. Sampling was carried out by gently scraping with a sterile blade and needle, and the resulting material was stored in screw-cap sterile specimen tubes. Axenic cultures of the organisms were obtained by dilution and repeated cultivation in agarized BG11 medium (Rippka et al. 1979). Single cell colonies were repeatedly picked and transferred to fresh medium, yielding several uni-cyanobacterial isolates. Enrichment and experimental cultures were grown in an orbital incubator (Gallenkamp, Loughborough, UK) at a temperature of 30 ± 1°C and continuous illumination of 2.18 W m^{-2} provided by MARCA cool white fluorescent tubes. For analysis, samples of the five strains were collected after culturing for 15 days, at the end of the exponential growth phase.

Light microscopy

Microscopic investigations of fresh samples were performed using a Zeiss Axioskop (Germany) light microscope, and images were taken using a Nikon Coolpix 5000 digital camera. After staining with alcian blue solution in 3% acetic acid (Ramus 1977) observations were made with a Reichert-Jung Polyvar photomicroscope (Wien, Austria). Both microscopes were equipped with differential interference contrast.

Identification of the strains

For morphological identification of unstained cyanobacterial strains, the diacritic traits used for description of botanical species were considered (Anagnostidis and Komarek 1990; Komárek and Anagnostidis 1989, 1999, 2005). Due to the morphological simplicity of the cultured strains, provisional identification based on phenotypic characters was obtained. However, DNA was extracted from the P2b and P2n strains using the GeneMATRIX plant and fungal DNA purification kit (EURx) according to the manufacturer's instructions. The 16S rRNA gene was amplified using the cyanobacterial specific primer CYA359 (41) as the forward primer and the universal primer C (5-ACGGGCGGTGTGT AC-3), corresponding to *Escherichia coli* positions 1406 to 1392, described in Bruno et al. (2009). After purification from the agarose gel using a Qiaquick gel extraction kit (Qiagen), the PCR products (1100 bp) were cloned into pGEM-T Easy vector (Promega) and sequenced using primers CYA359 and C.

Isolation of polysaccharides

The polysaccharides released and solubilized in the culture medium (released polysaccharide-RPS) were obtained by centrifuging the cultures at $3000 \times g$ for 15 min. The supernatant was then dialyzed (12–14,000 Da cut off, Medicell International Ltd, London) for 24 h against distilled water (changed every 8 h) in order to remove salts in the medium and small particulates. Polysaccharides were precipitated from the supernatants by adding 97% cold ethanol and desiccated by air-drying.

Determination of anionic density

Aliquots of cultures were dialyzed for 24 h against distilled water before being centrifuged at $3000 \times g$ for 15 min. Anionic density was evaluated both on pellets and on the RPS fraction solubilized in the supernatant, following the method described by Ramus (1977), as modified by Bar-Or and Shilo (1987).

Determination of calcium, magnesium and iron (II) removal

Cyanobacterial cultures, confined in natural cellulose dialysis membranes (12-14,000 Da cut off), were

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dialyzed for 24 h against distilled water (changed every 8 h) in order to remove salts present in the culture medium. The dialysis tubing containing the confined biomass was dipped into solutions containing calcium and magnesium or iron metal (10 ppm) solutions for 24 h. As a control, dialysis membranes without cyanobacterial cells, and previously dialyzed for 24 h against distilled water, were dipped in 10 ppm solutions of each metal. The amount of metal removed by the blank and the cyanobacterial biomass was measured by atomic adsorption spectrometry (SpectrAA plus, Varian Inc, CA, USA). Specific metal removal (q), expressed as mg of metal removed per g of biomass dry weight, was calculated using the following equation (Volesky and May-Phillips 1995):

$$q = V(C_b - C_s)/m$$

where V is the sample volume (l), C_b and C_s are respectively the metal concentration in the blank and the metal concentration (mg l^{-1}) in the sample after the contact with the biomass and m is the biomass dry weight (g). The tests were performed in triplicate and the resulting standard deviations (SDs) are reported in the results.

Determination of the monosaccharidic composition of the RPS fraction

Aliquots of RPS were hydrolyzed with 2 N trifluoroacetic acid at 120°C for 120 min and then cooled on ice. Samples were ultrafiltered with centrifugal filters (Amicon Ultra-4) at 8200 × g for 20 min, dried in a rotary evaporator and analyzed by using a Dionex ICS-2500 ion exchange chromatograph (Sunnyvale, CA), according to the method reported by Micheletti et al. (2008).

UV exposure experiments

Cultures for UV exposure experiments were previously acclimated in Pyrex flasks under continuous irradiation at 2.18 W m^{-2} white light (VIS), produced by cool-white fluorescents bulbs devoid of any UV emission (0 W m^{-2} UVA, 0 W m^{-2} UVB). Aliquots of exponentially growing cultures were put in uncovered Petri dishes with BG-11 medium (Rippka et al. 1979) at 30°C and pre-treated for 3 days under three different light conditions: (i) 2.18 W m^{-2} VIS only, (ii) 2.18 W m^{-2} VIS plus 3.20 W m^{-2} UVA (peak at 360 nm), (iii) 2.18 W m^{-2} VIS plus 1.36 W m^{-2} UVA and 2.96 W m^{-2} UVB. After the three different pre-treatments, four sub-sets of all cultures were finally irradiated for 1 h, at constant temperature, with 5.13 W m^{-2} UVC, provided by a germicidal lamp emitting at 260 nm, 2.18 W m^{-2} VIS, 0.14 W m^{-2} UVA, or 0.17 W m^{-2} UVB. Test cultures were exposed to UV radiation, whereas control cultures were covered with Pyrex lids to avoid irradiation.

UV-screening, pigment extraction and quantification

Samples (~50 mg dry weight) of the tested cultures were extracted overnight with 100% acetone and extracts clarified by centrifugation. Absorbance was measured at 384 nm, where scytonemin contributes the maximum absorbance, using a MARCA UV-Vis spectrophotometer. Absorbance measurements were also performed at 490 nm and 663 nm to determine the contribution of carotenoids and chlorophyll *a*, respectively. Correction of the residual scatter was performed by subtracting the absorbance at 750 nm from every measured absorbance. Scytonemin absorbances were corrected using the trichromatic equations of Garcia-Pichel and Castenholz (1991).

For extraction of mycosporine-like amino acids (MAAs), samples of ~50 mg dry weight were extracted with 25% (v/v) methanol from 1 to 2 h. The specific content was obtained by measuring the absorbance at 334 nm, the maximum absorbance of MAAs, and by correcting the value using the equation by Garcia-Pichel and Castenholz (1993). Scytonemin and the MAA contents were expressed as units of absorbance. One A equals the value of the absorbance when the amount of compound is dissolved in 1 ml of solvent and measured with 1-cm-path-length cuvette. Corrected absorbances were normalized to dry weight (A_λ g^{-1}).

Chlorophyll fluorescence measurements

In order to evaluate the physiological status of the cyanobacterial strains under changed radiance conditions, photosynthetic activity was determined by measuring the variable chlorophyll (chl *a*) fluorescence of photosystem II (PSII). The effects of ultraviolet radiations UVA, UVA + UVB, and UVC on PSII in terms of electron transport rate (ETR), were estimated using a portable fluorometer Mini-PAM/F (Pulse-Amplitude-Modulation, Walz, Germany). The PAM probe was placed perpendicularly at a distance of ~10 mm from the sample to yield a measurable signal from the cells. The saturating light pulse corresponded to a photosynthetic photon flux density (PPFD) of 5300 μmol m^{-2} s^{-1} with a duration of 0.8 s. Measured light was about 0.3 μmol m^{-2} s^{-1}, and the actinic light 10 μmol m^{-2} s^{-1}. For quenching analyses, the actinic light was directed to the sample using the same probe.

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Results

Characterization and identification of isolates

Five cyanobacterial strains were isolated, three (temporarily named P2a, P2b and P2n) from the biofilm collected from the walls of Parasurameswar Temple and two (temporarily named P3 and P4) from the Khandagiri caves. Three strains were unicellular chroococcalean species (P2b, P2n and P4), while the others were filamentous oscillatorialean taxa. The morphological features of the coccoid strains allowed them to be identified as members of the genera *Gloeocapsosis* (P2b and P2n) and *Gloeocapsa* (P4), while the two thin filamentous strains belonged to the genera *Leptolyngbya* (P2a) and *Plectonema* (P3) (Figure 1). Strains P2b and P2n were similar to *Gloeocapsosis* cf. *crepidinium* (Figure 1b–d, h, i) with blue-green sub-sphaerical, irregularly rounded cells, 3–7 μm in diameter, densely aggregated in colonies, surrounded by unlamellated sheaths in groups of 2-4–16 cells. However, preliminary molecular data obtained for the strains P2b and P2n (data not shown) suggested that they might belong to the genus *Chroogloeocystis*, with 98% sequence similarity of the 16S rRNA gene to a strain of *Chroogloeocystis siderophila* isolated from microbial mats in hot springs (Brown et al. 2005). Strain P4 was identified as *Gloeocapsa* cf. *kuetzingigiana* (Figure 1f, m) because of the gelatinous thallus, light blue-green cells 2.5–3.0 μm wide, with colourless and scarcely lamellated sheaths; cells in 2–4–8 group-forming colonies. All the chroococcalean strains occurred as blackish biofilm on the sandstone walls of Hindu temples in the Bhubaneswar area.

The *Leptolyngbia* strain P2 (Figure 1a, g) had thin trichomes, surrounded by colorless firm sheaths open at the end, and blue-green pigmented cells longer than wide, 1.3–1.5 μm in diameter. The *Plectonema* strain P3 (Figure 1f, l) had an olive-green thallus, filaments with colourless sheath, trichomes forming false branches and cells around 3.5 μm in diameter.

These species occurred in biofilms on exposed surfaces either of the Parasurameswar temple or Khandagiri caves.

Light microscope observations after staining with alcian blue showed a dense blue coloration surrounding the cells of all the five cyanobacteria (Figure 1g–m). In the case of strains P2b and P4, the dye also bound to the material released into the medium, suggesting the presence of anionic compounds, both in the layer

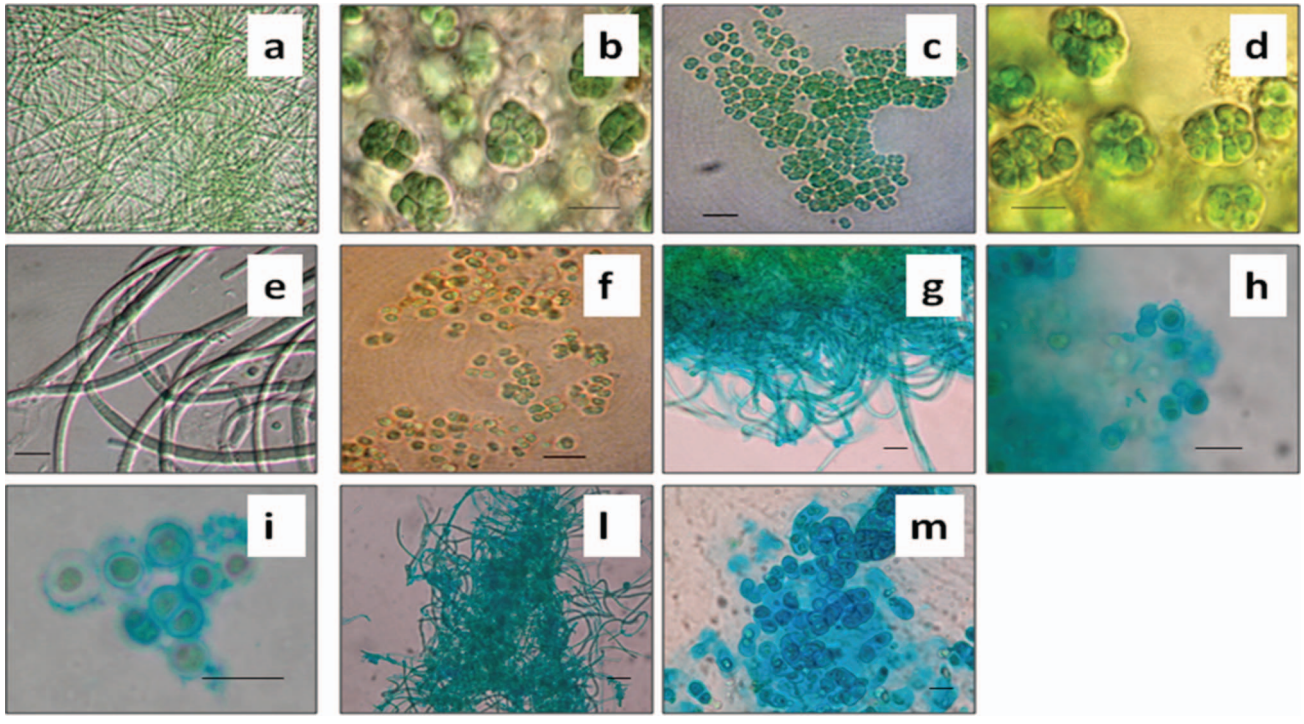

Figure 1. Light microphotographs of the four cyanobacterial strains in culture (a–f) and after staining with alcian blue (g–m). (a) *Leptolyngbya* sp.(strain P2a); (b) *Gloeocapsosis* cf. *crepidinum* (strain P2b); (c, d) *Gloeocapsopsis* cf. *crepidinium* (strain P2n) at early and late developmental stage; (e) *Plectonema* sp. (strain P3); (f) *Gloeocapsa kuetzingigiana* (strain P4); (g) *Leptolyngbya* sp. (strain P2a); (h) *Gloeocapsosis* cf. *crepidinum* (strain P2b); (i) *Gloeocapsopsis* cf. c*repidinium* (strain P2n); (l) *Plectonema* sp. (strain P3); (m) *Gloeocapsa kuetzingigiana* (strain P4). Scale bars = 10 μm.

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tightly attached to the cells and in the solubilized RPS found in the culture medium. The direct measurement of the anionic charge density on the pellet (ie cells plus sheath) and in the supernatant (ie solubilized RPS) of the cultures of the five strains showed that in strain P3, a negative charge was mostly present on the external cell layer, while in the case of strains P2b and P2n, the negative charge seemed to be more concentrated in the released polysaccharides (Table 1). Strains P2a and P2n showed the lowest amount of charge over their whole biomass.

Monosaccharidic composition of RPSs

Ion exchange chromatography of the hydrolyzed RPSs showed that the polysaccharides produced by strains P2a, P2n, P3 and P4 were composed of 12 different monomers, while the RPS produced by strain P2b was composed of 11 types of sugar (Table 1). The aldohexose glucose was present in large quantity, representing 20–42% of the moles of sugar in the RPSs. The ketohexose fructose was found in all the RPS except that produced by strain P2b. The aldopentoses, xylose, ribose and arabinose, were always found, the former representing from 11–24% of the moles of sugar. The deoxy-sugars rhamnose and fucose were also found, the latter being the sugar present in the highest amount in the RPS produced by strain P2b. The acid sugars, glucuronic and galacturonic acids, and glucosamine were found in the polysaccharides released by all the five strains.

Capability of the strains to bind calcium, magnesium and iron (II)

The five cyanobacterial strains showed a good affinity for the divalent ions tested, with different values for the specific removal q of each metal (Figure 2). Strains P2b and P3 showed the highest specific affinity for calcium and magnesium, the former being the metal removed from solutions at the highest q value. On the other hand, strain P2a showed the lowest q values for the three metals compared to the other strains. Except for strain P2n, which showed a similar q value for calcium and magnesium (8.13 ± 5.28 and 9.16 ± 1.46 mg g^{-1}, respectively), the other strains showed q values for Ca^{2+} two to four times higher than for Mg^{2+}. Strain P2n showed a high affinity towards Fe^{2+}, showing a q value 30 times higher than the ones shown by the other strains and 9 times higher than its q values for Ca^{2+} and Mg^{2+}.

Effects of UV radiation on photosystem II activity

All five strains showed a complete inhibition of electron transfer from photosystem II to photosystem I when exposed to UVC radiation for 1 h (Figure 3). However, after pre-treatment for 3 days with UVA or UVB, strains P2a, P2b, P2n and P3 showed an improved tolerance to UVC, as they still had partial

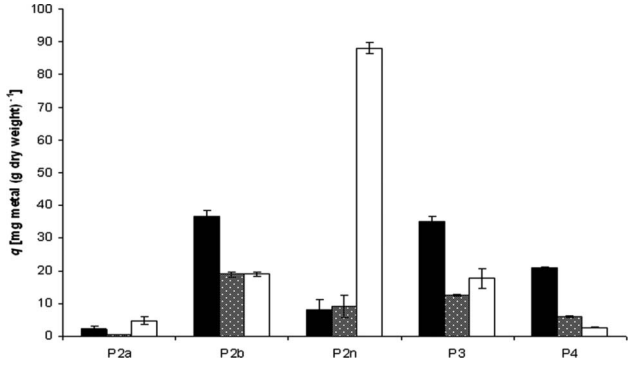

Figure 2. Specific metal removal q (expressed as mg of metal adsorbed g^{-1} of dry biomass) of the five strains towards Ca^{2+} (black bars), Mg^{2+} (gray bars) and Fe^{2+} ions (white bars). All values are the mean of at least three replicates $\pm$ SD.

Table 1. Monosaccharide composition (expressed as micromoles of monosaccharide per gram of RPS dry weight) of the released polysaccharides (RPS) and the anionic density of the released polysaccharides and the residual biomass (pellet) of the five cyanobacterial strains.

	Monosaccharides (μmoles [g dry wt]$^{-1}$)*												Anionic density[a]	
	Fuc	Rha	GalN	Ara	GlcN	Gal	Glc	Xyl	Fru	Rib	GalAc	GlucAc	RPS**	Pellet**
P2a	9.3	5.0	1.3	1.2	0.4	0.7	28.0	10.0	4.2	8.3	11.4	0.2	0.23 $\pm$ 0.08	0.12 $\pm$ 0.03
P2b	74.0	4.5	3.5	5.9	3.7	10.0	26.0	4.5	n.d.	3.0	6.3	20.2	0.91 $\pm$ 0.16	0.10 $\pm$ 0.02
P2n	4.4	6.9	5.9	1.3	14.5	10.9	78.0	44.3	1.9	3.2	7.4	8.2	0.27 $\pm$ 0.09	0.10 $\pm$ 0.03
P3	9.7	1.3	0.2	4.0	3.6	13.0	22.0	10.0	2.0	2.3	12.0	0.1	0.13 $\pm$ 0.04	0.48 $\pm$ 0.12
P4	7.0	13.0	0.16	30.0	5.0	10.0	30.0	30.0	0.9	6.7	12.0	3.1	0.24 $\pm$ 0.10	0.15 $\pm$ 0.04

Note: Fuc = fucose; Rha = rhamnose; GalN = galactosamine; Ara = arabinose; GlcN = glucosamine; Gal = galactose; Glc = glucose; Xyl = xylose; Fru = fructose; Rib = ribose; GalAc = galacturonic acid; GlucAc = glucuronic acid; n.d. = not detected. *Data shown are mean values from at least three determinations; SDs never exceeded 5%. [a]$n = 3 \pm$ SD. **= mg of alcian blue fixed/(mg dry weight)$^{-1}$.

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photosynthetic activity compared to cultures exposed to UVC without any pre-treatment. No improvement in the ETR value was only found for strain P4 following UVA and UVA + UVB pre-treatments. After UV irradiation, all the strains except P2n, showed lower ETR values compared to cultures exposed only to VIS light (control cultures), whereas P2n showed a higher photosynthetic capacity after UV radiation with an ETR_{max} of 14.3 measured after exposure to UVC after UVA + UVB pre-treatment.

The P2a control culture reached photoinhibition at a PFD value of 350 μmol (photons) m^{-2} s^{-1}, corresponding to an ETR value of 12.9 μmol (electrons) m^{-2} s^{-1}. When acclimated to UVA or UVA + UVB and then exposed to UVC, the strain still showed photosynthetic activity although the measured ETR_{max} value was between 2.1 and 3.8-fold lower compared to control samples treated with single and double irradiation, respectively. Control cultures of strain P3 reached photoinhibition at a PPFD of 150 μmol m^{-2} s^{-1}, corresponding to an ETR_{max} value of 7.7 μmol m^{-2} s^{-1}. Also in this case, the exposure to UV radiation resulted in a decrease in photosynthetic activity compared to the control culture. Values of PPDF exceeding 55 μmol m^{-2} s^{-1} caused photoinhibition and an ETR_{max} reduction of 1.7 and 2.2-fold compared to the control for UVA and UVA + UVB treated cultures, respectively. Strain P4 showed the lowest values in terms of ETR. Photoinhibition was reached at a PPFD of 100 μmol m^{-2} s^{-1} with an ETR max of 3.9 μmol m^{-2} s^{-1}. For strain P2b, UVA exposure did not cause photoinhibition even at the highest actinic light tested, reaching values of ETR of about 8 μmol m^{-2} s^{-1}, while in the case of irradiation with UVA + UVB, a decrease in ETR was observed at PPFD of 210 μmol m^{-2} s^{-1}, corresponding to an ETR_{max} of 6.4 μmol m^{-2} s^{-1}.

UV screening pigments after exposure to UV irradiation

Pigment extraction and quantification revealed that exposure to UV radiation caused an increase in absorbance at those wavelengths that correspond to the absorption maxima of the sunscreen compounds MAAs and scytonemin (Figure 4). An increase in both corrected absorbances at 384 (scytonemin) and 334 nm (MAAs) was observed for strains P2a, P2n and P3, while P2b and P4 showed an increase only at 334 nm.

Pre-treatment with UVA always caused an increase in the corrected absorbance at 334 nm, whereas only strains P2a, P2n and P3 reacted similarly at 384 nm. The double exposure (UVA followed by UVC) compared to a single UVC exposure, resulted in a further increase in the corrected absorbance at 334 nm in the case of strains P2n and P4, while it caused a

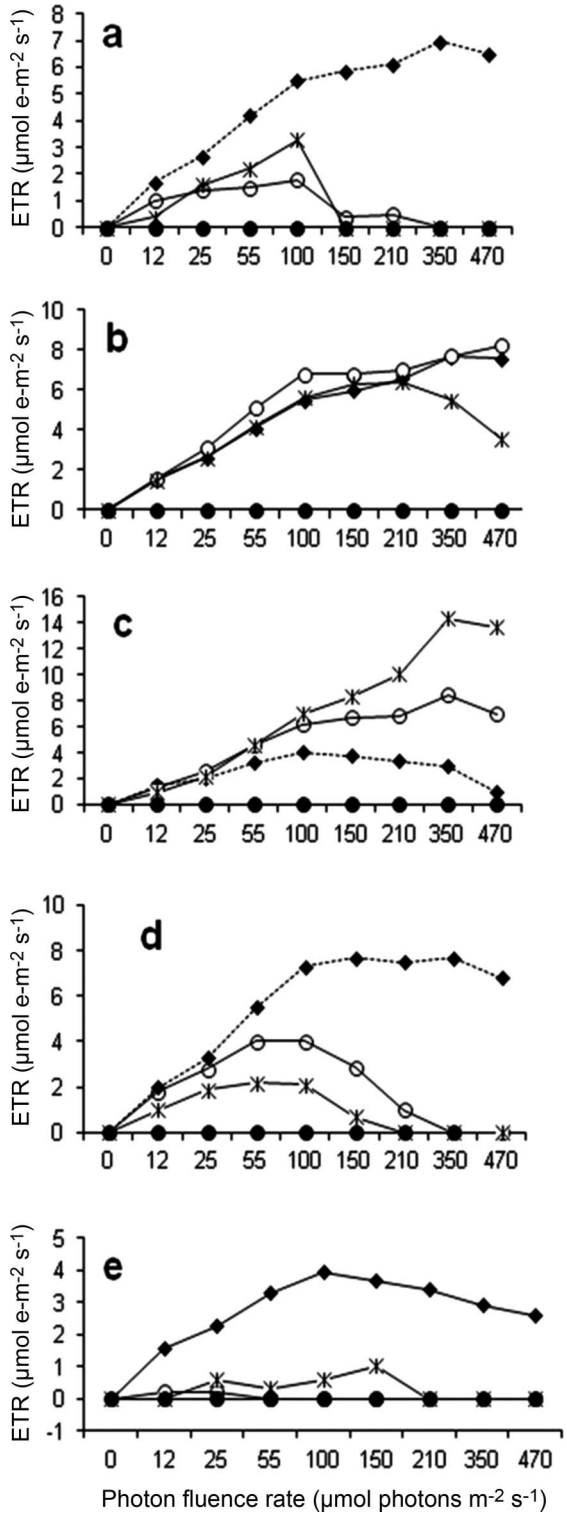

Figure 3. Electron transport rate *vs* irradiance (μmol photons m^{-2} s^{-1}) of strains P2a (a), P2b (b), P2n (c), P3 (d) and P4 (e) after UVC exposure for 1 h. To is the control, ie no exposure to UV; pretreatment with UVA (open circles) and UVA + UVB (crosses) was for 3 days. —◆— To, —○— PostUVC after UVA pretreatment, —✳— PostUVC after UVA + UVB pretreatment, —●— Direct exposure to UVC.

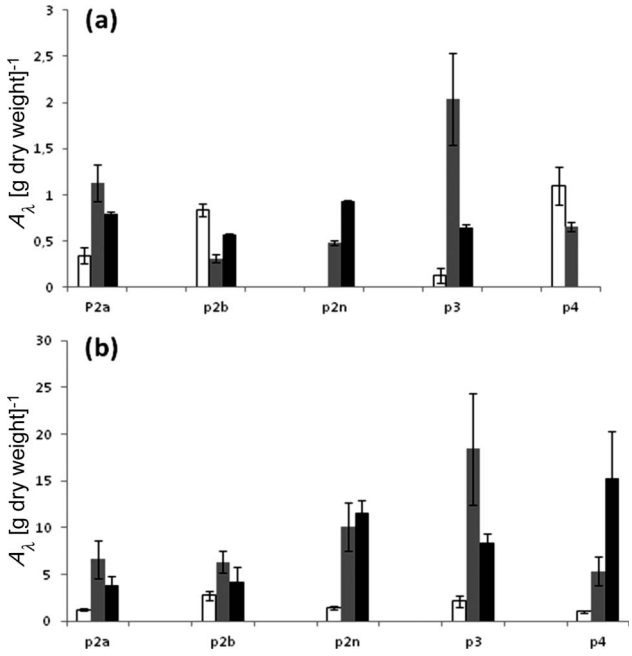

Figure 4. Corrected absorbance at 384 nm (where scytonemin contributes maximum absorbance) after 100% acetone extraction (a) and corrected absorbance at 334 nm (where MAAs contribute maximum absorbance) after 25% methanol extraction (b). Specific contents are expressed as A_λ [g dry weight]$^{-1}$. □ Control, ■ after UVA, ■ after UVA + UVB.

decrease in the corrected absorbance in the other cases. UVA + UVB exposure did not elicit further scytonemin synthesis as no significant increase in the corrected absorbance at 384 nm was observed compared to UVA irradiation, though a decrease was observed in the case of strain P2a, P3 and P4.

Discussion

The exopolysaccharides produced by the five strains of cyanobacteria isolated from the biofilms growing on the walls of the Parasurameswar temple and Khandagiri caves were composed of at least eleven different sugars. According to the data available regarding cyanobacterial polysaccharides, only 8% of these polymers have been characterized by 10 or more different monosaccharides (De Philippis et al. 2001; De Philippis and Vincenzini 2003; Pereira et al. 2009). Thus, it appears that the complexity of the heteropolymers produced by the five cyanobacteria could give specific advantages to the microorganisms residing in the biofilms growing on the monuments. The presence of a large number of different monosaccharides represents a considerable trophic resource for the community embedded in the EPS matrix of the biofilm when polymers are degraded. Furthermore, the RPS of the five Indian strains were composed of hydrophobic and hydrophilic fractions, which may facilitate different interactions with the surrounding environment. Indeed, the deoxy-sugar rhamnose, which ranged from 6–9% of the total moles of polymeric sugars in all the RPS studied, and fucose, which ranged from 5–12 moles %, are both sugars known to confer hydrophobic properties to polysaccharides (Bellezza et al. 2003), thus increasing the cohesiveness of the biofilm and its capacity to adhere to solid surfaces (Nielsen et al. 1997). On the other hand, the rather high anionic density found in the external layers of polysaccharide of the five cyanobacteria studied is capable of conferring hydrophilicity on the polymers. This is due to the presence of uronic acids, which were found in all the RPS fractions, ranging from 8–28 moles % of the total moles of monosaccharides. Indeed, uronic acids are known to contribute both to the anionic nature and to the sticky character of polysaccharides (Arias et al. 2003; Decho et al. 2005; Mancuso et al. 2005). The simultaneous hydrophobic and hydrophilic features of the polysaccharides in the slimy layer surrounding the cells are capable of facilitating both the adhesion of the biofilm to the stone surfaces of the monuments and the storage of the water when available, thus providing the cyanobacteria and associated microorganism with a survival strategy in periods of drought. The hydrophilic nature of the sugars is also responsible for ionic bonds with charged molecules such as metal cations, especially Ca^{2+}, Mg^{2+}, and Fe (II) (Brown and Lester 1982; Pierson and Parenteau 2000; Mancuso et al. 2005).

Under laboratory conditions, all five cyanobacteria showed the capability of binding calcium, magnesium and iron, thereby removing them from aqueous solutions. It was also found that the specific metal uptake q for Ca and Mg was directly related to the total anionic density measured for cyanobacterial biomass. This result strongly suggests that the interactions of metal/biomass are mainly based on ionic forces developed between the negative charged molecules present in the external layers of the exopolysaccharide and the metal cations. However, this direct correlation was not found for the removal of Fe (II): the highest value of q was shown by strain P2n, which showed one of the lowest values of anionic density. This contradictory result might be explained if the ability to chelate metal ions is related not only to the number of negatively charged groups present on the RPS, but also to their distribution on the macromolecule and their accessibility (Arias et al. 2003; de Kerchove and Menachem 2008).

The stones utilized for the construction of the Parasurameswar temple and the rocks where the Khandagiri caves were dug are mainly composed of

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SiO_2 (67.33%) and Al_2O_3 (18.86%). Calcium, magnesium and iron (II) represent respectively the 0.5%, 1.02% and 4.01% of the minerals in the rocks. The affinity shown by the RPS of the five cyanobacteria towards Ca^{2+}, Mg^{2+} and Fe^{2+} may provide the microorganisms residing in the biofilm with metals for cell metabolism and stabilization of the biofilm structure, as was previously suggested (Welch and Vandevivere 1994). Metal cations can also be captured after mineral dissolution of the stones caused by acidic processes and by the presence of acidic polysaccharides, which can weaken metal-oxygen bonds in the minerals and bind trace metals at the same time. It is known that mineral dissolution cannot be enhanced by polymers mainly composed of neutral sugars (Mac Leod et al. 1994; Chen et al. 1995; Wolfaardt et al. 1999). Moreover, the accumulation of iron in the cyanobacterial capsule and sheath may provide a reserve in the case of low iron availability (Pierson et al. 1999) as well as providing the cells with protection against the harmful effects of high irradiation, in some cases coupled with scytonemin (Pierson et al. 1993; Pierson and Parenteau 2000; Soule et al. 2009; Varnali and Edwards 2010). It is worth stressing that strain P2n, one of the two cyanobacterial strains that showed a 98% sequence similarity of the 16S rRNA gene with a strain of *Chroogloeocystis siderophila*, was characterized by a high resistance to Fe (Brown et al. 2005) and of the five isolates under study, showed the highest *q* value towards this metal.

The five strains were shown to be capable of colonizing high light-exposed environments by acquiring resistance to intense solar radiation. Exposure to low intensity radiation was shown to be sufficient to improve the tolerance of four out of the five strains to subsequent harsher radiation. The results strongly suggest that UV protective pigments were secreted. Extraction procedures with methanol and acetone after UV exposure showed increases in the corrected absorbance at 334 nm and 384 nm, respectively, which are most likely related to the synthesis of protective pigments such as MAA-like substances and scytonemin, respectively. Many studies reported in the literature have shown that synthesis of scytonemin or MAAs can be induced by UVA and/or UVB irradiation (Ehling-Schulz et al. 1997; Oren and Gunde-Cimerman 2007). In the present case, while an increase in absorbance at 334 nm (corresponding to MAAs) was always observed after UVA and/or UVA + UVB irradiation, a significant increase in absorbance at 384 nm (corresponding to scytonemin) was only observed in some of the cyanobacteria studied. Scytonemin synthesis, if it took place, was shown to be elicited by UVA irradiation, whereas no additional synthesis was shown to be elicited by double irradiation. In addition, the improvement in photosynthetic activity after the acclimation treatment has to be ascribed to the contribution of both pigments, with MAAs providing compensation when scytonemin synthesis was minimal or absent. The protective action of MAAs is explained both as a UV-absorbing compound and as a scavenger of oxygen-species coming from the ionizing effect of high energy irradiation (Holzinger et al. 2009).

In the case of strain P4, the acclimation pre-treatment both with single and double irradiation did not lead to an improvement in UVC tolerance. Despite a prominent increase in the corrected absorbance at 334 nm after UVC irradiation (after the double irradiation pre-treatment), no significant improvement in the physiological state of the culture was observed in comparison to the non-acclimated culture. The absence of scytonemin may have been the cause, as it is the only case in which corrected absorbance at 384 nm wavelength showed a sharp decrease after UVA pre-treatment and it was not detectable after UVA + UVB treatment.

UV-acclimated P2n cells were the only ones that showed a higher photosynthesis rate in comparison with cultures exposed only to visible wavelengths. The highest ETR value was obtained with UVA + UVB pre-treatment. Similar results were obtained by Holzinger et al. (2009) with the green alga *Zygonema*, collected in Ny-Alesund, Spitsbergen, Norway and exposed to natural environmental stress from UV radiation. The acclimation plasticity of strain P2n was confirmed by the corrected absorbances related to secretion of both scytonemin and MAAs that showed a sharp increase from single to double irradiation pre-treatment. Natural cyanobacterial communities and the diazotrophic heterocystous *Hassallia byssoidea* (ex *Tolypothrix byssoidea*) isolated from sandstone temples in Orissa showed even higher concentration of these sun-screen pigments (Roy et al. 1997; Adhikary and Sahu 1998).

In conclusion, even if the strains under study represent only a part of the phototrophic community residing on the lithic surfaces of the monuments, it is worth stressing that when grown under laboratory conditions, they showed specific acclimation properties that conferred the capability of coping with the high solar radiation typical of tropical countries.

The polysaccharide slime released by the five strains grown under laboratory conditions showed high chemical variability and complexity, which provided the polymeric matrix of the biofilm with both hydrophobic and hydrophilic characteristics. If the same macromolecular and chemical features are maintained when the polymer is synthesized under natural conditions, the biofilm will have the versatility

required for attachment and stability when exposed to wind and water erosion. The affinity for bivalent cations enhances the capacity of cells to bind and store trace metals, thus providing an advantage for survival in harsh environments, but it also contributes to weakening of mineral substrata and enhancing stone decay.

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