Archives of Microbiology (2023) 205:32
https://doi.org/10.1007/s00203-022-03368-w

BRIEF REPORT

*aro*F and *cm*2: potential molecular markers for the detection of stone-inhabiting Actinobacteria on cultural heritage sites

Samah Mohamed Rizk[1,2,3] · Mahmoud Magdy[2] · Filomena De Leo[3] · Olaf Werner[1] · Mohamed Abdel-Salam Rashed[2] · Rosa María Ros[1] · Clara Urzì[3]

Received: 9 August 2022 / Revised: 8 October 2022 / Accepted: 6 December 2022 / Published online: 19 December 2022

Abstract

Tangible archeological sites and stone monuments are naturally decayed and deteriorated over time, providing substances that can sustain life, although they provide a complicated ecosystem characterized by low nutrition and desiccation. Stone-inhabiting bacteria (SIB) and especially members of the phylum Actinobacteria dominate such environments, particularly the members of the family Geodermatophilaceae. We used the published data of two confirmed SIB species to mine their genomes for specific molecular markers to rapidly survey the presence of SIB in cultural heritage material prior to further analysis. The search focused on the mycosporine-like amino acids (MAAs) synthesis pathway. MAAs are intracellular compounds biosynthesized by the shikimic acid pathway to synthesize aromatic amino acids and were found related to abiotic resistance features in microorganisms. Based on genome mining, the DAHP II (*aro*F) and a homolog of the Chorismate mutase gene (*cm*2) were found mostly in Actinobacteria and few other species. After calibration on five stone-inhabiting Actinobacteria (SIAb) species using conventional PCR, newly designed primers were successfully applied to environmental DNA extracted from two Egyptian pyramidal sites using a qPCR approach. This is the first report of *aro*F and *cm2* as qPCR markers to detect SIAb from cultural heritage material prior to proceeding with further analysis (e.g., metagenomics and meta-barcoding analyses).

Keywords Stone-dwelling bacteria · Cultural heritage microbiology · qPCR-based approach

Introduction

Archeological sites and stone monuments represent a valuable and important part of the cultural heritage worldwide. A large percentage of the world's tangible cultural heritage is made from stone, and it is slowly but irreversibly disappearing by the natural transformation into sand and soil in a process essential to sustain life on earth (Allsopp et al. 2004; Gadd 2017). Despite the harsh conditions resulting from low water availability and nutrient concentration, stone surfaces represent a complex ecosystem that enables a diverse range of microorganisms to proliferate. Besides algae, lichens, and fungi, cyanobacteria, actinobacteria, and other bacteria were reported to form the complex microflora of the stone surface (Urzi et al. 2001; Scheerer et al. 2009; Pena-Poza et al. 2018). Stone-inhabiting bacteria are peculiar bacterial species, especially members of the phylum Actinobacteria (SIAb) that mostly dominate this habitat type (Stackebrandt et al. 1997; Ding et al. 2022). Within Actinobacteria, species of the family Geodermatophilaceae were previously reported as SIB (Urzi et al. 2001; Ennis et al. 2022), causing a deteriorating aspect on stones in outdoor conditions (e.g., colored spots as well as biopitting and powdering) (Urzì and Realini 1998; Sghaier et al. 2016). Those species can resist UV light, ionizing radiation, desiccation, and heavy metals (Rainey et al. 2005; Gtari et al. 2012; Montero-Calasanz et al. 2014, 2015). Their isolation in vitro is not easy, and they are often underestimated because the cultivation method

Communicated by Erko Stackebrandt.

✉ Mahmoud Magdy
m.elmosallamy@agr.asu.edu.eg

[1] Department of Plant Biology, Faculty of Biology, Murcia University, 30100 Murcia, Spain

[2] Department of Genetics, Faculty of Agriculture, Ain Shams University, 11241 Cairo, Egypt

[3] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98166 Messina, Italy

needs at least 15 days of incubation and appropriate nutritional media (Urzi 2004). Therefore, DNA- and RNA-based methods have provided a faster and more reliable approach and have gained much attraction over the past decade in the study of the biodeterioration of cultural heritage (Sterflinger 2010; Otlewska et al. 2014; Piñar et al. 2020). The primary use of these methods was detecting and characterizing microorganisms in environments that are notoriously hard to culture and where the inhabited microorganisms are often underestimated (Scheerer et al. 2009). Besides the difficulties of sample collection in a non-destructive way from culturally important sites (Urzì and De Leo 2001) and the low concentration of the extracted eDNA (Schneegurt et al. 2003), the identification techniques can be expensive and time-consuming. For such reasons, many researchers use Epifluorescence microscopy to detect the microbial community's viability in samples before proceeding with further analysis (e.g., metagenomics and meta-barcoding analysis) (Ricca et al. 2020). Therefore, to have a fast and reliable molecular approach for surveying cultural heritage material for possible biodeterioration agents would be an essential tool in the field of applied cultural heritage microbiology.

Mycosporine-like amino acids (MAAs) are a family of intracellular compounds biosynthesized by the shikimic acid pathway to synthesize aromatic amino acids (Wada et al. 2015) and have been described in Actinobacteria (Miyamoto et al. 2014). They have an ampholyte nature and high denaturation temperature with water-soluble properties (Bozkurt and Kara 2017) and are expressed under abiotic stresses, e.g., solar radiation (Bhatia et al. 2011) and extreme conditions (Rosic and Dove 2011). Key genes related directly or indirectly to the MAAs synthase pathway would be promising single-copy molecular markers to detect SIAb species adapted to stone surfaces' harsh conditions.

Results and discussion

The pathway analysis showed that both the MAAs and shikimate pathways (i.e., biosynthesis of folates and aromatic amino acids) start by stereospecific condensation of phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate (E4P) to give rise to Phospho-2-dehydro-3-deoxyheptonate (DAHP) using Phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase), and then convert to 3-dehydroquinate (DHQ) by 3-dehydroquinate synthase (*aro*B) gene, followed by either 3-dehydroquinate dehydratase (*aro*Q) to redirect the DHQ to 3-dehydroshikimate for shikimate pathway, or O-methyl transferase (OMT) to redirect the DHQ to deoxygadusol for the MAAs pathway (Fig. 1). Based on STRING-db, the *aro*B and *aro*Q genes were found not only in the two referenced SIAb genomes (*Blastococcus saxobsidens* DD2 and *Geodermatophilus*

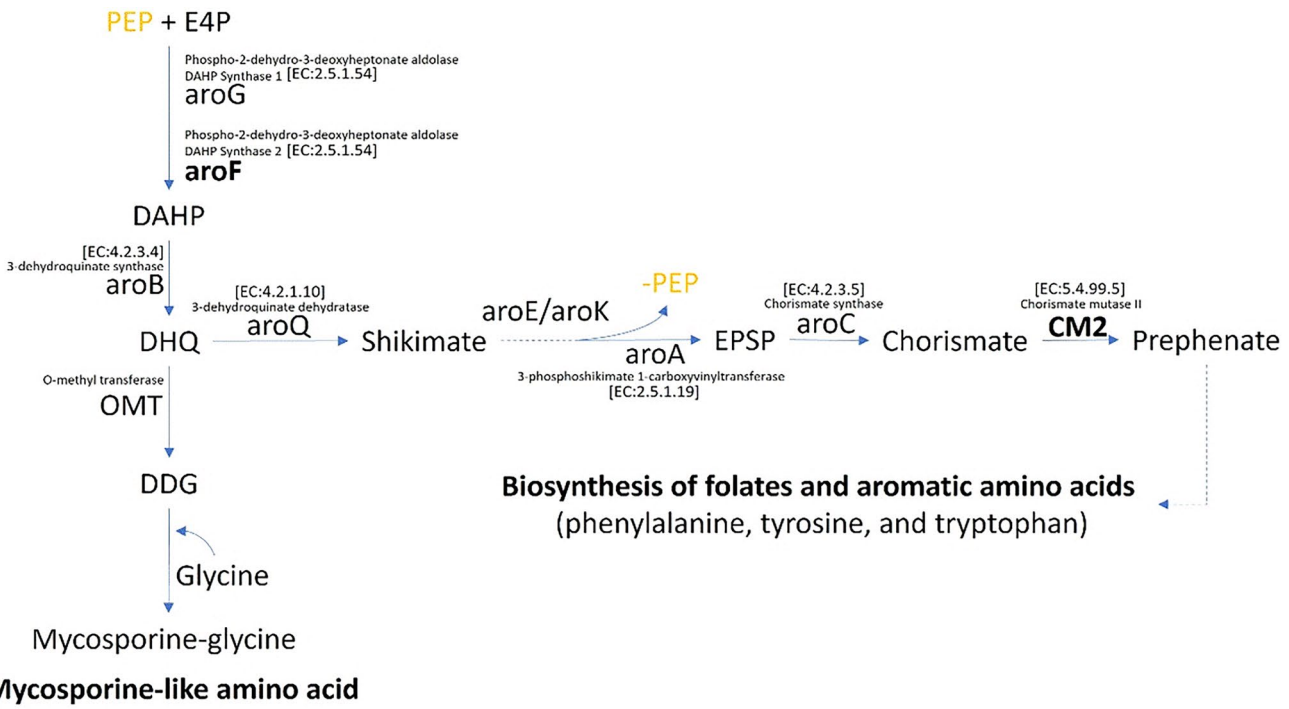

Fig. 1 Mycosporine-like amino acids and shikimate (biosynthesis of folate and aromatic amino acids) schematic pathway based on literature and STRING information. Potential candidate genes identified from one of the two SIAb, *Blastococcus saxobsidens*, are written in bold

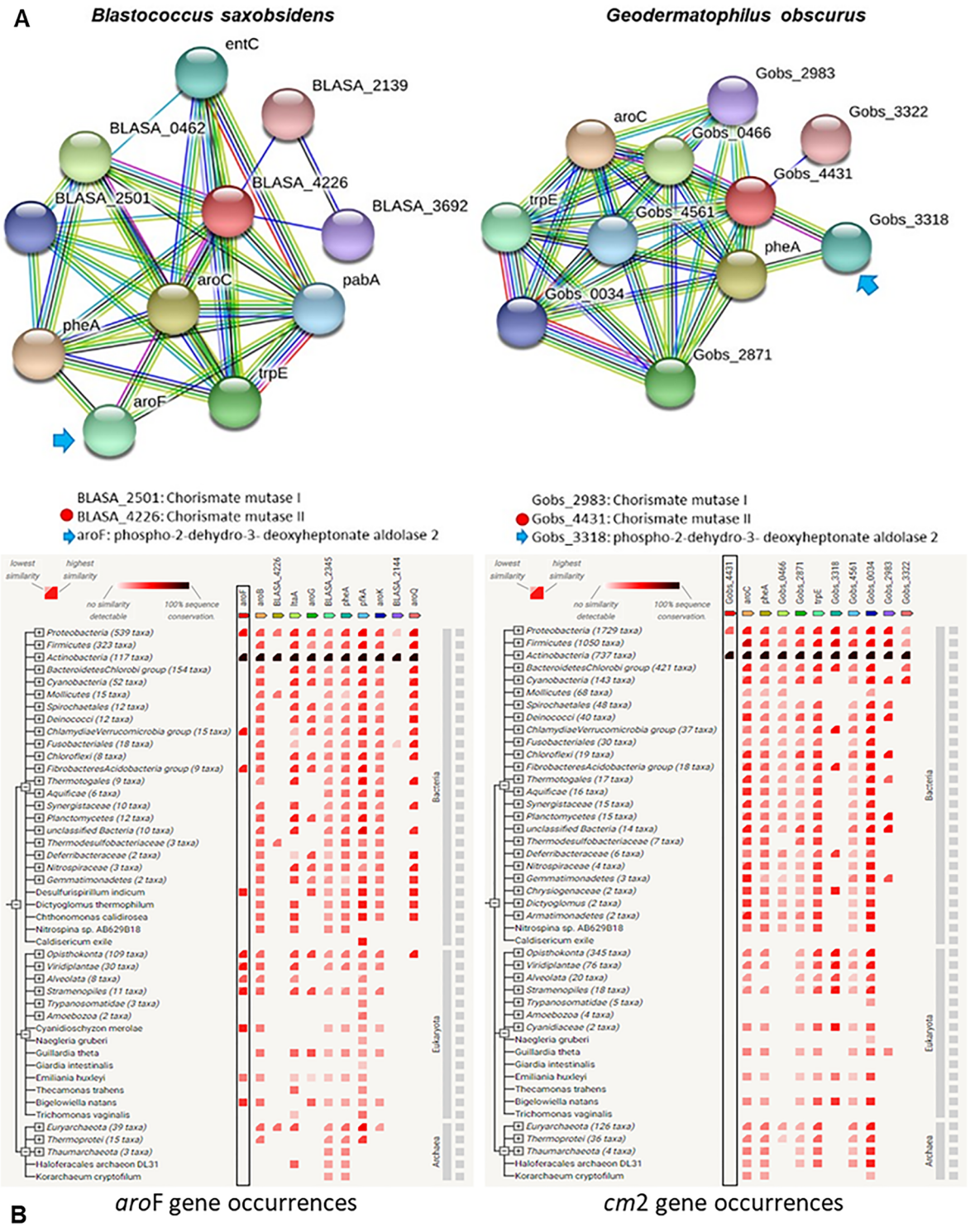

Fig. 2 Gene interaction and occurrences for *aro*F and *cm*2 genes in two SIAb species (*Blastococcus saxobsidens* and *Geodermatophilus obscurus*). **A** Gene interaction network visualized based on Chorismate mutase gene for the two stone-inhabiting bacteria. Blue arrow points to the *aro*F gene, while the red circle represents the *cm*2 gene. **B** The candidate genes occurrence graph along with neighbor genes and homologs from the same pathway among different organisms based on the STRING database. The search targeted the *B. saxobsidens* DD2 (BLASA) *aro*F gene (left) and the *G. obscurus* DSM 43160 (Gobs) *cm*2 gene coded as Gobs_4431 (right)

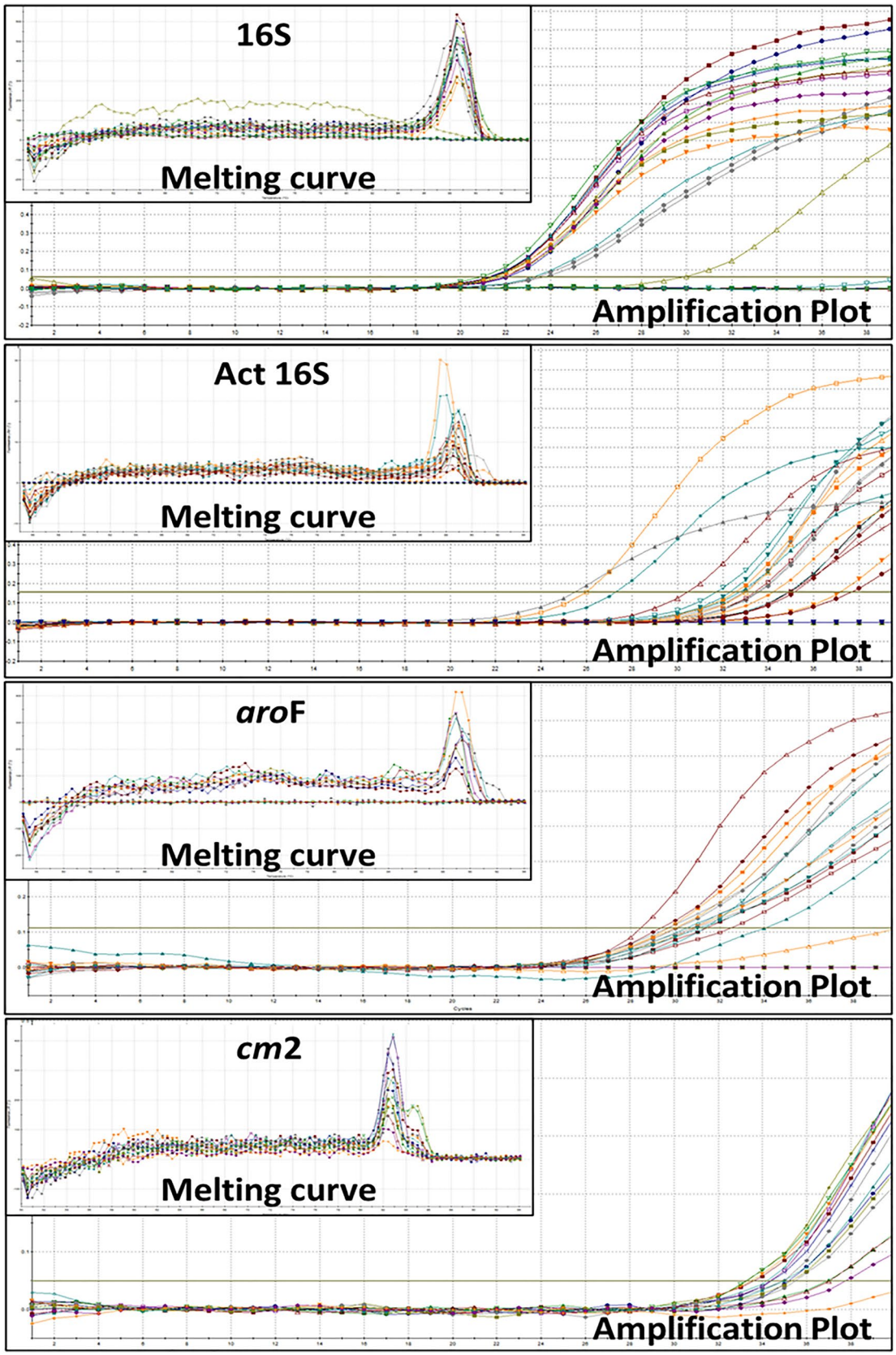

Fig. 3 qPCR amplification plots and melting curves for 16S rRNA universal qPCR primer (16S), Actinobacteria-specific 16S rRNA primer (Act 16S), *aro*F and *cm*2 using the eDNA extracted from cultural heritage material

Table 1 List of the newly designed qPCR primers, melting temperature (Tm), expected product size (bp), target gene, and reference if previously published

Organism group	Primer ID	5'-Seq-3'	Tm	bp	Gene	References
Universal	926F	AAA CTC AAA KGA ATT GAC GG	50	136	16S rRNA	De Gregoris et al. (2011)
	1062R	CTC ACR RCA CGA GCT GAC				
Actinobacteria	Act664F	TGT AGC GGT GGA ATG CGC	60	277	16S rRNA	Yang et al. (2015)
	Act941R	AAT TAA GCC ACA TGC TCC GCT				
Actinobacteria/proteobacteria	aroFqF	GCG CAC ATC GAC TTC ATC TC	57	283	*aro*F	Current study
	aroFqR	CGA AGT GCC GGG TCT TGT AG				
Actinobacteria	CmutqF	GTA ACA GCA CTG GCA GC	57	237	*cm*2	Current study
	CmutqR	CGA TCT CGT CTA TCC GCT CC				

obscurus DSM 43160) but further in many other organisms. Additionally, the OMT was not found in the two SIAb genomes, which disqualifies those genes as potential SIAb molecular markers. The DAHP synthase gene was found in duplicate as DAHP synthase I (*aro*G) and II (*aro*F), the latter of which was found only in few species belonging to Proteobacteria, Actinobacteria, Chlamydiae-Verrucomicrobia group, Fibrobacteres-Acidobacteria group, *Desulfurispirillum indicum*, and some eukaryotic organisms. The Actinobacteria were the highest in terms of similarity and number of species when *aroF* from *B. saxobsidens* was used as an inquiry (Fig. 2). Based on the *aroF* interactive gene network, an additional candidate from the same pathway was detected, namely Chorismate mutase, which was found in two homologs, homolog I (*cm*1) and II (*cm*2). Like *aro*F, the *cm*2 was found exclusively in Actinobacteria and few species from Proteobacteria (e.g., *Escherichia coli*, Fig. 2). Based on *E. coli* co-expression data from the same database, *aroF* was found to co-express with *cm2* (known as *phe*A in *E. coli*; *r* value = 0.85).

New qPCR primers were designed and optimized for single-band amplification using cPCR approach on five actinobacteria species. The ancient Djoser and Lahun pyramids were chosen to validate the elucidated markers. They are among the oldest and largest pyramids in the Memphis necropolis of ancient Egypt. Deterioration was observed in many parts of both pyramidal complexes in the form of dark spots, coloration, and brittle rocks. The universal 16S rRNAs as well as the newly designed qPCR primer were amplified successfully from 12 archeological samples collected evenly from the two pyramids and generally varied among the collected samples (Fig. 3). The estimated $MR_{SIAb}R$ was 1.01 ± 0.04 and 0.98 ± 0.05 for *aro*F and *cm*2, respectively, reflecting an equal efficiency of the two potential markers as the Actinobacteria-specific 16S rRNA primers to amplify the Actinobacteria successfully. Our results supported the *aro*F and *cm*2 potentiality as molecular markers to detect SIAb specimens' presence in cultural heritage material. The use of the qPCR is for its sensitivity to detect very low concentrations, as qPCR achieves low limits of detection (1×10–7 ng/µl) and a higher detection rate employed in eDNA for laboratory and field samples than cPCR (Xia et al. 2018). To the best of our knowledge, most of the qPCR protocols to detect and/or quantify bacterial communities in cultural heritage material were designed based on the multi-copy 16S rRNA gene (Mondo et al. 2019; Salazar et al. 2006; Urzi et al. 2004). The developed approach was designed to rapidly detect the presence of SIAb but not for their absolute quantification. The qPCR amplification of those marker increases the probability to detect Actinobacteria from this type of samples, but not necessarily be evidence for their inhabiting activity and thus, require further isolation and identification. This protocol is developed as a preliminary test for archeological stone samples and cultural heritage material prior to metagenomic and/or culture-dependent analyses to reduce cost and time, respectively.

Materials and methods

The MAAs synthesis pathway was investigated using STRING online tools (Szklarczyk et al. 2019). Along with previous reports, a more comprehensive pathway was constructed using the two confirmed Geodermatophilaceae SIAb (*B. saxobsidens* DD2 (BLASA) and *G. obscurus* DSM 43160 (Gobs)). Candidate genes were extracted from the genomes using Geneious Prime (Kearse et al. 2012) and aligned with copies from Actinobacteria species acquired by BLAST search for qPCR primer design (Table 1). All the qPCR primers were calibrated using conventional PCR (cPCR) applied to five Actinobacteria species isolated from stone, three species belong to Geodermatophilaceae (*B. saxobsidens* DSM 44509 (Urzì et al. 2004), *G. carrarae* DSM 44511 (Urzi et al. 2001), and *Modestobacter* sp. DSM 44446 (Urzi et al. 2001)) and two species belong to Micrococcaceae, *Kocuria sediminis* DPS08B and *Micrococcus*

luteus DPS11B. The latter two strains were isolated from our previous work on the Djoser pyramid area among other Actinobacteria (members of Geodermatophilaceae, Micrococcaceae, and Planococcaceae; unpublished data) following Urzi et al. (2001) and Girish et al. (2022). DNA extraction from the bacterial strains was performed using PureLink® Genomic DNA kit (Cat# K182001, Thermo Fisher Scientific, USA) according to the kit protocol. DNA quality was checked using 1% (w/v) agarose gel electrophoresis, visualized by pre-added RedSafe® dye under UV light, and quantified using Quantus™ Fluorometer (Promega, USA). The cPCR reactions were performed using MyTaq™ Red Mix (Cat# BIO-25043, BioLine, UK) kit. In each 25 µl reaction tube included 5 pmol of each primer, and 40 ng on template DNA were added. The amplification was carried out using Techne™ 512 thermocycler (Techne, UK). PCR programs were adjusted according to the primer pair melting temperature (Tm) as follows: the first denaturation step at 95 °C for 3 min was followed by denaturation at 95 °C for 20 s, annealing was set according to each primer pair (Table 1), extension at 72 °C for 30 s. The last three steps were repeated 34 times, with a last extension 72 °C for 5 min. PCR products were visualized using 1.5% agarose gel electrophoresis.

Environmental DNA (eDNA) from 12 archeological samples collected evenly from two pyramids (Djoser and Lahun pyramids) was extracted using MOBIO Powersoil DNA isolation (Qiagen, Germany). The eDNA was used for the newly developed qPCR markers validation. The universal primers for 16S rRNA (De Gregoris et al. 2011) and the Actinobacteria-specific 16S rRNA (Yang et al. 2015), along with the newly designed primers were calibrated and tested using the following recipe and qPCR conditions: 5 pmol of each primer, 1× of SensiFAST™ SYBR® Lo-ROX Kit (Bioline, UK), 1 ml of eDNA and H_2O up to a total volume of 20 µl were prepared for each sample. The qPCR was performed using Stratagene Mx3000P qPCR machine (Agilent Technologies, USA) and was set to the default normal 3-steps standard thermal profile, while the annealing temperature was adjusted to 57 °C for *aro*F and *cm*2. The marker efficiency to amplify SIAb ($MR_{SIAb}R$) was evaluated for each sample using the following equation: $MR_{SIAb}R$ = relative Actinobacteria CT value (CT16S/CTAct)/relative marker CT value (CT16S/CTmarker), where the CT (cycle threshold) values are for the 16S universally amplified bacteria (CT16S), 16S amplified Actinobacteria (CTAct), and amplified bacteria with a gene copy of the tested marker (CTmarker).

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SMR and MM. Bacterial strains and resources were provided by SMR and CU. Supervision on the work was done by OW and RMR in Spain, FL and CU in Italy, and MM and MA-SR in Egypt. The fund acquisition was done by MM and MA-SR. The first draft of the manuscript was written by SMR and MM and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding The experiments were partially funded by Science and Technology Development Fund (STDF-Egypt) project no. 26383.

Availability of data and materials All data generated or analyzed during this study are included in this published article.

Code availability No coding was required for this work.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval The study doesn't require ethical approval because the samples used in this study didn't include any human participants, cell lines or related specimen.

Consent to participate The study doesn't require consent to participate because the samples used in this study didn't include any human participants, cell lines or related specimen.

Consent for publication The study doesn't require consent for publication because the samples used in this study didn't include any human participants, cell lines or related specimen.

References

Allsopp D, Seal KJ, Gaylarde CC (2004) Introduction to Biodeterioration, 2nd edn. Cambridge University Press

Bhatia S, Sharma K, Sharma A et al (2011) Mycosporine and mycosporine-like amino acids: a paramount tool against ultra violet irradiation. Phcog Rev 5:138. https://doi.org/10.4103/0973-7847.91107

Bozkurt HS, Kara B (2017) Bifidobacterium and mycosporin-like amino acid cooperation: a new era for intestinal diseases treatment? J Gastric Disord Ther 3:2

De Gregoris TB, Aldred N, Clare AS, Burgess JG (2011) Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. J Microbiol Methods 86:351–356

del Mondo A, de Natale A, Pinto G, Pollio A (2019) Correction to: Novel qPCR probe systems for the characterization of subaerial biofilms on stone monuments. Ann Microbiol 69:1097–1106

Ding X, Lan W, Yan A et al (2022) Microbiome characteristics and the key biochemical reactions identified on stone world cultural heritage under different climate conditions. J Environ Manag 302:114041

Ennis NJ, Dharumadurai D, Tisa LS (2022) Draft genome sequences of 6 actinobacterial strains isolated from rock surfaces obtained from Indian stone ruins in Tamil Nadu, India, and rocks from New England, United States. Microbiol Resour Announc 11:e00024-e122

Gadd GM (2017) Geomicrobiology of the built environment. Nat Microbiol 2:16275. https://doi.org/10.1038/nmicrobiol.2016.275

Girish S, Ravi L, Krishnan K (2022) Isolation of stone-dwelling Actinobacteria. In: Dharumadurai D (ed) Methods in Actinobacteriology. Springer, New York, NY, US, pp 163–168

Gtari M, Essoussi I, Maaoui R et al (2012) Contrasted resistance of stone-dwelling Geodermatophilaceae species to stresses known

to give rise to reactive oxygen species. FEMS Microbiol Ecol 80:566–577. https://doi.org/10.1111/j.1574-6941.2012.01320.x
Kearse M, Moir R, Wilson A et al (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649. https://doi.org/10.1093/bioinformatics/bts199
Miyamoto KT, Komatsu M, Ikeda H (2014) Discovery of gene cluster for mycosporine-like amino acid biosynthesis from Actinomycetales microorganisms and production of a novel mycosporine-like amino acid by heterologous expression. Appl Environ Microbiol 80:5028–5036
Montero-Calasanz MC, Hofner B, Göker M et al (2014) *Geodermatophilus poikilotrophi* sp. nov.: a multitolerant actinomycete isolated from dolomitic marble. BioMed Res Int 2014:1–11
Montero-Calasanz MC, Hezbri K, Göker M et al (2015) Description of gamma radiation-resistant *Geodermatophilus dictyosporus* sp. nov. to accommodate the not validly named Geodermatophilus obscurus subsp. dictyosporus (Luedemann, 1968). Extremophiles 19:77–85
Otlewska A, Adamiak J, Gutarowska B (2014) Application of molecular techniques for the assessment of microorganism diversity on cultural heritage objects. Acta Biochim Pol. https://doi.org/10.18388/abp.2014_1889
Pena-Poza J, Ascaso C, Sanz M et al (2018) Effect of biological colonization on ceramic roofing tiles by lichens and a combined laser and biocide procedure for its removal. Int Biodeterior Biodegrad 126:86–94
Piñar G, Poyntner C, Lopandic K et al (2020) Rapid diagnosis of biological colonization in cultural artefacts using the MinION nanopore sequencing technology. Int Biodeterior Biodegrad 148:104908
Rainey FA, Ray K, Ferreira M et al (2005) Extensive diversity of ionizing-radiation-resistant bacteria recovered from sonoran desert soil and description of nine new species of the genus *Deinococcus* obtained from a single soil sample. Appl Environ Microbiol 71:5225–5235
Ricca M, Urzì CE, Rovella N et al (2020) Multidisciplinary approach to characterize archaeological materials and status of conservation of the Roman Thermae of Reggio Calabria site (Calabria, South Italy). Appl Sci 10:5106
Rosic NN, Dove S (2011) Mycosporine-like amino acids from coral Dinoflagellates. Appl Environ Microbiol 77:8478–8486
Salazar O, Valverde A, Genilloud O (2006) Real-time PCR for the detection and quantification of *Geodermatophilaceae* from stone samples and identification of new members of the genus *Blastococcus*. Appl Environ Microbiol 72:346–352
Scheerer S, Ortega-Morales O, Gaylarde C (2009) Microbial deterioration of stone monuments—an updated overview. Advances in applied microbiology. Academic Press, Netherlands, pp 97–139
Schneegurt MA, Dore SY, Kulpa CF (2003) Direct extraction of DNA from soils for studies in microbial ecology. Curr Issues Mol Biol 5:1–8
Sghaier H, Hezbri K, Ghodhbane-Gtari F et al (2016) Stone-dwelling actinobacteria *Blastococcus saxobsidens*, *Modestobacter marinus* and *Geodermatophilus obscurus* proteogenomes. ISME J 10:21–29
Stackebrandt E, Rainey FA, Ward-Rainey NL (1997) Proposal for a new hierarchic classification system, Actinobacteria classis nov. Int J Syst Bacteriol 47:479–491
Sterflinger K (2010) Fungi: their role in deterioration of cultural heritage. Fungal Biol Rev 24:47–55. https://doi.org/10.1016/j.fbr.2010.03.003
Szklarczyk D, Gable AL, Lyon D et al (2019) STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47:D607–D613
Urzi C (2004) Microbial deterioration of rocks and marble monuments of the Mediterranean basin: a review. Corros Rev 22:441–458. https://doi.org/10.1515/CORRREV.2004.22.5-6.441
Urzì C, De Leong F (2001) Sampling with adhesive tape strips: an easy and rapid method to monitor microbial colonization on monument surfaces. J Microbiol Methods 44:1–11
Urzì C, Realini M (1998) Colour changes of Notos calcareous sandstone as related to its colonisation by microorganisms. Int Biodeterior Biodegrad 42:45–54. https://doi.org/10.1016/S0964-8305(98)00045-6
Urzi C, Brusetti L, Salamone P et al (2001) Biodiversity of Geodermatophilaceae isolated from altered stones and monuments in the Mediterranean basin. Environ Microbiol 3:471–479
Urzì C, Salamone P, Schumann P et al (2004a) *Blastococcus saxobsidens* sp. nov., and emended descriptions of the genus Blastococcus Ahrens and Moll 1970 and *Blastococcus aggregatus* Ahrens and Moll 1970. Int J Syst Evol Microbiol 54:253–259
Urzi C, La Cono V, Stackebrandt E (2004b) Design and application of two oligonucleotide probes for the identification of Geodermatophilaceae strains using fluorescence in situ hybridization (FISH). Environ Microbiol 6:678–685
Wada N, Sakamoto T, Matsugo S (2015) Mycosporine-like amino acids and their derivatives as natural antioxidants. Antioxidants 4:603–646
Xia Z, Johansson ML, Gao Y et al (2018) Conventional versus real-time quantitative PCR for rare species detection. Ecol Evol 8:11799–11807
Yang Y-W, Chen M-K, Yang B-Y et al (2015) Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in mouse feces. Appl Environ Microbiol 81:6749–6756