# Esterase- and irradiation resistance- profiling of Geodermatophilaceae isolated from Sahara desert stones and monuments

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Running title: Actinobacteria isolated from stones and monuments of Tunisia and Egypt.

**Keywords:** Esterase, UV resistance, Gamma ray resistance, *Geodermatophilus*, *Blastococcus*, *Modestobacter*, 16S rRNA gene, PAGE, cationic strength.

**ABSTRACT** **(reformat accordingly to the journal style)**

**Aim:** To assess ~~genetic diversity and~~ esterase profiling of members of ~~actinobacteria~~ Geodermatophilaceae isolated from desertic stones and monuments in Tunisia and Egypt.

**Methods and Results:** Geodermatophilaceae ~~Actinobacterial~~ isolated from desert stones and monuments in Tunisia and Egypt, were assigned on the basis of partial 16S rRNA sequences to *Geodermatophilus* (14 strains), *Blastococcus* (2 strains) and~~,~~ *Modestobacter* (9 strains)~~,~~ ~~*Arthrobacter* (23 strains) and *Micromonospora* (9 strains)~~ genera. The isolates have been screened and typed based on a major group of esterase hydrolytic activit~~ies;~~y ~~esterase~~. Strains exhibited a divers and complex pattern of electrophoretic bands. Cluster analysis delineated five dissimilar groups with a higher heterogeneity mainly correlated to ~~ARDRA~~ the 16S rRNA gene analysis clustering. Esterase produced by member of ~~*Geoderamatophilaceae*~~*Geodermatophilaceae* family has an optimal activity around 40°C and at pH 8. Esterases from ~~*Geoderamatophilus*~~*Geodermatophilus* strains display a high resistance to thermal inactivation and alkaline pH and retained 30 and 20% of the maximum activity by heating for 20 min at 120°C and at pH 12 respectively and was completely inactivated after 30 min at 120°C.~~.~~ Enzyme have been activated strongly in the presence of $Ca^{2+}$ and $Mg^{2+}$ ions, moderately by $Zn^{2+}$ ions and markedly inhibited by $Cu^{2+}$ and $Co^{2+}$ ions.

**Conclusions:** ~~Beside~~ *Geodermatophilaceae* ~~genera~~isolates~~, stones and monuments in arid area of Tunisia and Egypt host other members of actinobacteria from *Arthrobacter* and *Micromonospora* genera. Almost actinobacterial isolates~~ share a rich and particular pool of esterase activities that could be directly linked to harsh conditions characterizing their ecological habitat including high-level of aridity, temperature and ionic strength and low nutrient availability.

**Significance and Impact of the Study:** *Geodermatophilaceae* ~~, *Arthrobacter* and *Micromonospora* are the main actinobacteria isolated from desert stones and monuments~~.

1 Esterase could be considered as enzymatic signature that outlines actinobacterial adaptability in

2 arid area.

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## INTRODUCTION

Stone surfaces are complex habitats in which environmental conditions resulting from solar radiation, temperature, aridness and lack of nutrients fluctuate widely (Gorbushina, 2007). These surfaces, however, have been shown to harbour a large variety of bacterial genera that demonstrate a high tolerance to such stressful factors. ~~Rock inhabitants include associations of fungi, cyanobacteria and heterotrophic bacteria (Urzi~~ Urzì ~~and Realini., 1998; Urzi~~ Urzì ~~et al., 2001; Gorbushina, 2007).~~ *Geodermatophilaceae* are among the main settlers of these stringent environments (~~Urzi~~ Urzì et al., 2001, 2004)~~ with other sympatric prevalent bacteria like *Arthrobacter* (Eppard et al., 1996) and some *Micromonospora* strains (Zezza et al., 1995; Urzi Urzì et Realini., 1998)~~. Members of these genera are preponderantly recovered from environments characterized by dry conditions including those extreme like Antarctic or hot desert soils (Mevs et al., 2000), rocks and monument surfaces (Eppard et al., 1996; Urzì and Realini, 1998; ~~Urzi~~ Urzì et al., 2001; Salazar et al., 2006). These actinobacteria have been associated to aesthetic alteration of stones like orange, black and grey stains and patinas as well as with mechanical damages, like biopitting and powdering proving their active involvement in decay process (Urzì et al., 2001).

~~Diversity of microbial communities found in stones and monuments surfaces has been studied according to different approaches such as (colony and cell morphology, physiology, protein patterns and 16S rRNA gene sequences studies (Salazar et al., 2006). Moreover,, electrophoretic polymorphism of proteins such as esterases is widelycould be used for differentiating bacterial species on the basis of their genetic relationships (Goullet et Picard, 1995). Thanks to their extent biochemical diversity and their great electrophoretic variability (Gillespie, 1991), esterase has proved its reliability to highlight genetic diversity of microbial communities.~~ Esterases ~~(EC 3.1.1.x)~~ represent a diverse group of hydrolases catalyzing the cleavage and formation of ester

bonds. They are widely distributed in animals, plants and microorganisms. Many *Geodermatophilaceae* ~~of them~~ show a wide substrate tolerance which led to the assumption that they have evolved to enable access to various carbon sources or to be involved in catabolic pathways (Bornscheuer, 2002), improving their surviving abilities in dry environments and favouring the final ~~. Thus esterase may be useful as biomarker of adaptation of *Geodermatophilaceae* and other bacterial isolates to the~~ colonization and alteration of stone and monuments.~~–~~ The aim of the present study is to assess the ~~diversity and the~~ esterase profiling of member of *Geodermatophilaceae* genera ~~and other prevalent sympatric actinobacteria~~ isolated from desert stones and monument in Tunisia and Egypt in comparison with the relative type strains.

## MATERIALS AND METHODS

### *Strains isolation and growth conditions*

Isolation and cultivation ~~procedure~~ of Geodermatophilaceae were as described by ~~Urzi~~ Urzì et al. (2001). Depending on the artistic extends of the sampled surface (monument, building or rock from quarry) 100 mg to 21 g of rock samples or powders from scraped surfaces were taken. Samples ~~was~~ were individually ground to a powder in a sterile mortar and suspended (1:10 w/v) in physiological saline (0.85% NaCl) supplemented with 0.01% (w/v) Tween 80. The suspension was stirred for 60 min and one millilitre of each suspension and its decimal dilutions were plated on Luedemann medium (Luedemann, 1968) supplemented with cycloheximid. Incubation was carried out at 28°C. Observation of the cfus was performed after 7, 15 and 30 days. Bacterial colonies were described morphologically, and those resembling the morphological features of ~~actinobacteria~~ Geodermatophilaceae were picked out and subcultured twice to three times~~ird~~ on Luedemann medium. *Geodermatophilus obscurus ssp. obscurus* (DSM 43160T) (Luedemann,

1968), *G. obscurus ssp. utahensis* (DSM 43162) (Eppard et al., 1996), *G. obscurus ssp. dictyosporus* (DSM 43161) (Eppard et al., 1996), *Geodermatophilus obscurus ssp. amargoseae* (DSM 43136), *Blastococcus aggregatus* (DSM 4725T) (Ahrens and Moll, 1970) *B.* ~~*Saxobsidens*~~ *saxobsidens* (DSM44509) (~~Urzi~~ Urzì et al., 2004) and *Modestobacter*~~—~~ *multiseptatus* (DSM 44406T) (Mevs et al., 2000) were used as reference strains.

***DNA extraction, ARDRA and 16S ~~rDNA~~ rRNA gene sequencing***

To extract DNA, bacterial isolates were grown at 30°C for 3 days on Luedemann medium. Three to five well-isolated colonies were washed three times in sterile physiological saline. Total genomic DNA was extracted by a CTAB-SDS lysis protocol (Ausubel et al., 1994). ARDRA profiles were determined using the following restriction enzymes: *Hpa*II, *Rsa*I and *Cfo*I (Amersham Pharmacia Biotech). The 16S rRNA gene PCR products were purified from PCR reaction mixtures using the QIAquick Wizard PCR purification Kit (Promega, Madison, and VVI, USA), according to manufacturer instructions. The sequences were determined by cycle sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Monza, Italy), and underwent fragment separation in an ABI Prism~~TM~~ 310 DNA sequencing as previously described (Gtari et al., 2004). Similarity matrix of 16S rRNA gene sequences with closest neighbours was calculated using RDP utilities (Ribosomal Database Project II: http://rdp.cme.msu.edu/html). The nucleotide sequences of 16S rRNA gene were aligned using ~~Clustalx~~ ClustalX (Thompson et al. 1994) and compared to reference strains. Phylogenetic tree ~~were~~ was achieved using MEGA version 3.1 (Kumar et al., 2004) and the neighbour-joining algorithm (Saitou and Nei 1987). Bootstrap values were determined from 1000 replicates (Felsenstein 1985).

~~***Nucleotide sequence accession numbers***~~

~~The EMBL Accession Numbers for the 16S rRNA gene sequences of actinobacterial isolates BMG5726, BMG5737, BMG5724, BMG5721, BMG5749, BMG5729, BMG575, BMG576, BMG5714, BMG571, BMG572, BMG573, BMG578, BMG5711, BMG5750, BMG5751, BMG5728, BMG5742, BMG5748, BMG577, BMG5718, BMG5713, BMG5716 and BMG5723 determined in this study are from ????? to ????? respectively.~~

***Extraction of cellular proteins and electrophoretic esterase profiling***

Extraction of cellular proteins was performed as described by Ouzari et al. (2006). Cells were grown at 30°C for 3 days on Luedemann medium, washed twice with distilled water and resuspended in 400 µl of Tris buffer (20 mmol$^{-1}$ Tris, 100 mmol l$^{-1}$ KCl, 5%, w/v glycerol, [pH 7.5]). The cells disruption was carried out by sonication for 4 min at 50 W under cooling. The resulting cellular extract was centrifuged at 20 000 g for 30 min. The total protein content of the supernatant was evaluated using the Bradford method (Bradford, 1976). Ten micrograms of the supernatant ~~was~~ were analysed in 12% native PAGE using Tris-glycine buffer (Sambrook et al. 1989). After migration, the gel was equilibrated at 4°C in 0.15 mol l$^{-1}$ potassium phosphate buffer (pH 6.5) for 20 min. The staining procedure was performed as described by de Carvalho et al. (2003) incubating the gel for 30 min with gentle agitation in potassium phosphate buffer containing α-naphthyl acetate (α-NA), β-naphthyl acetate (β-NA), α-naphthyl propionate (α -NP) or β-naphthyl propionate (β -NP) (1%~~,~~ w/v in acetone) as substrates. Esterase activity signals were visualized as deep purple or black bands in the gel after incubation, in darkness, with the staining solution (1% w/v fast Garnet GBC salt in KP buffer). The evaluation of electrophoretic esterase profile was repeated ~~two times~~twice for each strain on different cellular extracts.

Esterase activity was determined by measuring the amount of p-nitrophenol released during enzymatic hydrolysis of different p-nitrophenyl esters. The release of p-nitrophenol was

continuously monitored at 410 nm using Biorad spectrophotometer (…..). Unless otherwise indicated, in a standard assay, esterase activity was measured with 0.2 mm p-nitrophenyl acetate (pNP-C5) as a substrate in 50 mm citrate-phosphate buffer (pH 7) containing 1% isopropanol at 70°C. Stock solutions of p-nitrophenyl esters were prepared by dissolving substrates in isopropanol. After preincubation, the reaction was started by adding enzyme to the reaction mix. One unit of esterase activity was defined as the amount of protein releasing 1 lmol.min$^{-1}$ of p-nitrophenol from pNP-C5. Measurements were corrected for background hydrolysis in the absence of enzyme. Measurements were carried out at least three times and the molar extinction coefficient of p-nitrophenol was determined for every condition prior to each measurement. Activity was determined from the initial rate of the hydrolysis reaction. The protein concentration was measured at 280 nm using a Biorad Spectrophotometer.

**pH, temperature and cationic strength effects**

The effect of pH on esterase activity was studied by measuring activities on p-nitrophenyl valerate for a pH range of 4.0–9.5. The buffers used were 50 mm citrate-phosphate (pH 4.0–8.0) and 50 mm Caps buffer (pH 9.5). The effect of temperature on esterase activity was studied in the range 45–95°C using 1 mm p-nitrophenyl valerate in the standard assay. The pH of the buffers was set at 25°C, and temperature corrections were made using their temperature coefficients (….) 0.0028 pH.°C$^{-1}$ for citrate-phosphate buffer and (0.018 pH.°C$^{-1}$ for CAPS buffer) (Beynon and Easterby, 2003). Effect of different metal ions ($Mg^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$) on enzyme activity was studied by incubating the enzyme with buffer in presence of 10-20 mM concentration of different metal ions and after 1 h of incubation at 30°C enzyme activity was assayed at 410 nm.

***Nucleotide sequence accession numbers***

The EMBL Accession Numbers for the 16S rRNA gene sequences of ~~actinobacterial~~Geodermatophilaceae isolates BMG5726, BMG5737, BMG5724, BMG5721, BMG5749, BMG5729, BMG575, BMG576, BMG5714, BMG571, BMG572, BMG573, BMG578, BMG5711, BMG5750, BMG5751, BMG5728, BMG5742, BMG5748, BMG577, BMG5718, BMG5713, BMG5716 and BMG5723 determined in this study are from ????? to ????? respectively.

## RESULTS

### *Identification of actinobacterial isolates*

~~Actinobacteria~~Geodermatophilaceae-like isolates and the characteristics of their origins of isolation, the rocky substrate, were listed in table 1. In total we isolated ~~57~~?? strains from 10 (???) stones and monuments located between Tunisia and Egypt. Amplified Ribosomal DNA Restriction Analysis (ARDRA), ~~were~~ performed on ~~57~~ the isolates and on the 9 *Geodermatophilaceae* reference strains, was done using three restriction enzyme *Hpa*II, *Rsa*I and *Cfo*I— to obtain a significant sampling of the sequences (Andreoni et al., 2000). Forty five different haplotypes with different incidence among the analysed strains have been detected. Thirty five haplotypes were represented each by one isolate, five by 2 isolates, two by 3 isolates and three haplotypes were represented respectively by 4, 5 and 7 isolates (Figure 1). Partial sequencing of 16S~~s~~ ~~rDNA~~ rRNA genes has been processed on 24 representative haplotypes whereas those from reference strains were retrieved from public database. Neighbour-Joining phylogenetic tree represented in Figure 1 permitted to assigned 24 isolates into three *Geodermatophilaceae* genera ~~(43%)~~: *Geodermatophilus* (~~24~~??%), *Blastococcus* (~~3~~??%) and *Modestobacter* (~~15~~??%) ~~while the 32 remaining isolates were clustered with *Arthrobacter* (40% of the isolates) and *Micromonospora* (15% of the isolates)~~.

***Esterase profiling of the isolates***

Esterases have been characterized on PAGE for the ~~57~~ ?? isolates belonging to *Geodermatophilus*, *Blastococcus*, and *Modestobacter*~~, *Arthrobacter* and *Micromonospora*~~ genera with the nine reference strains belonging to *Geodermatophilaceae* family (Figure 2). Most of the strains exhibits a complex esterase pattern~~s~~ (Figure 3). Much variation in number, colour, intensity and electrophoretic mobility is noted among strains of the same genus and those of the different studied genera. Strains showed two, three or multiple esterase bands (2≥ n ≥13) that hydrolysed substrates with different affinity; red colour bands are active against $\beta$-naphthyl propionate, those with dark brown colour are specific to both $\alpha$ and $\beta$-naphthyl acetate. Band intensity reflects enzyme activity; weak and strong intensity indicate reduced or high activities on a given substrate. A total of 30 polymorphic esterase bands active against $\alpha$ and $\beta$ -naphthyl esters of short chain fatty acids were distinguished assigning the strains to a 47 haplotypes. Thirteen haplotypes were found to include strains with high or perfect homology among them 10 haplotypes involve strains with complete homology in their esterase patterns, each one is represented by 2, 3 or 4 strains. The remaining haplotypes represent individual strains or group of strains with a low level of similarity (≤~~<~~ 0.4). Cluster analysis allowed the delineation of five dissimilar groups corresponding to the ~~five~~ three genera *Geodermatophilus*, *Blastococcus*~~,~~ and *Modestobacter*~~, *Arthrobacter* and *Micromonospora*~~ previously identified on the basis of 16S ~~rDNA~~ rRNA gene partial sequencing (Figure 3).

***Characterization of crude esterase activity*** ~~***in Geodermatophilaceae strains***~~.

Esterase activity was determined on *Geodermatophilaceae* reference strains and two isolates belonging to *Geodermatophilus* genus (BMG575 and BMG576) using p-nitrophenyl acetate as substrate. Temperature and pH effects on crude esterase activity were given in Figure 4 and

~~figure~~ Figure 5 respectively. The highest esterase activities are noted among members of *Geodermatophilus* genus as compared to those of *Blastococcus* and *Modestobacter* that show a relatively faint activity of their esterase. An optimal esterase activity was obtained around 40°C and at pH 8 for all strains. Esterase from *Blastococcus* is active between 20 and 50°C and in a wide pH range from 5 to 12. This activity is kept at pH ranging from 4 to 12 and rapidly decreased above 60°C for *Modestobacter* strains. Esterase activities from *Geodermatophilus* strains display a high resistance to thermal inactivation and alkaline pH. Enzyme activities were maintained for temperature ~~and pH~~ higher than 100°C and retained 30 and 20% of the maximum activity by heating for 20min at 120°C and at pH 12 respectively. Enzymes were completely inactivated after 30 min at 120°C. The effect of metal ions on *Geodermatophilaceae* esterase activity was tested using various metal ions: $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$ and $Co^{2+}$ at concentrations of 10, 30 and 50 ~~Mm~~mM. An inhibitory effect was observed with both concentrations of 30 and 50 mM, while at 10 mM.~~,~~ produced esterase have been activated strongly in the presence of $Ca^{2+}$ and $Mg^{2+}$ ions, moderately by $Zn^{2+}$ ions and markedly inhibited by $Cu^{2+}$ and $Co^{2+}$ ions (~~tableau~~ Table 2).

**DISCUSSION**

~~The biodiversity and the occurrence of actinobacteria in rocky substances have been studied over~~We isolated 57(check) of *Geodermatophilaceae* strains from 51 samples ~~,~~of Tunisian desert stones and Egypt and Tunisian monuments. All these samples were characterized by a high deterioration pattern described as black and grey diffused patinas and by phenomena of detachment, crumbling and powdering, tipically due to *Geodermatophilaceae* colonization (Urzì et al., 2001; 2004). Molecular analysis of the diversity of the isolates allowed the identification of the three *Geodermatophilaceae* genera *Geodermatophilus*, *Blastococcus* and *Modestobacter*.

Among the isolated strains, two BMG5737 and BMG5749 recovered from crumbly materials have been affiliated to *Blastococcus* genus~~ suggesting that members of this genus are less frequent on material surfaces and they rather colonize the internal parts of the rocks.~~. The majority of the isolates have been classified in the *Geodermatophilus* and *Modestobacter*.

~~-~~All these genera are known for their advanced ability of adaptation to extreme environments (Eppard et al., 1996; Mevs et al., 2000). ~~Indeed~~For example, they possess a well defined pleomorphism characterized not only by a transition from coccus to rod shape (~~luedemann, 1968~~Ishiguro and Wolfe, 1970; 1974; Mevs et al., 2000) but also by the presence of a thick wall and elaboration of dark coloured pigments that makes them particularly adapted to survive under stressful conditions related to high temperature, dryness and low nutrients availability. Moreover, it has been reported that actinobacteria deprived of an aerial mycelium such as those resembling to the family ~~*Geodermatophilus*~~ *Geodermatophilaceae* demonstrate a relatively marked genotypic and phenotypic diversity on exposed and arid rock surfaces~~.~~ (Eppard et al., 1996, Urzì et al., 2001).~~ Pigments formed by these microorganisms are the main factors in the formation of unesthetic blackening patinas (Urzi Urzì et al., 1992). Therefore, since it appears that among *Geodermatophilaceae* genera, *Modestobacter* and *Geodermatophilus* strains seem to permanently host stone surfaces and monuments with marble and calcareous substrates, it can be hypothesized that *Blastococcus* strains may occurhave a different ecological niche, such as frequently in the inner parts of crumbly materials, this result confirms the findings of Brussetti et al., 2008 that ascribed the predominance of *Blastococcus* strains in the depth.~~

~~With the aim to study the genomic diversity and relationship among the isolated actinobacteria and the reference strains, e~~The esterase enzyme ~~electrophoresis~~profiling ~~, known to be a powerful tool for assessing genetic relationships between and within species (Goullet et Picard., 1995),~~

~~have been applied.~~showed an high degree of genetic ~~Much~~ variation ~~in esterase profiles was observed~~ among strains.

This ~~Heterogeneity associated the complexity of patterns~~ was directly related to a large isoenzymatic dissimilarity among strains of the same genus and those of different genera~~, –~~. Moreover, ~~it revealed the~~this degree of variation of the enzymatic system ~~and~~ proved that the genetic structures of *Geodermatophilaceae* are extremely diverse. Several bacteria, such as isolates of *Rhizobium leguminosarum* (Young et al., 1987) and *Frankia*, the close related genus to *Geodermatophilaceae* within *Frankineae* sub-order (Gardes et al., 1987, Normand, 2006b), have been reported to have a large amount of variation as well.

Cluster analysis performed on electrophoretic esterase profiles on native polyacrylamide gel well highlighted the bacterial population diversity with 47 different haplotypes identified for 61 investigated strains, five groups were revealed according to the ARDRA and 16S ~~rDNA~~ rRNA gene sequencing previously carried out. Combining the data obtained with these molecular approaches, 26 haplotypes are selected and gathered into three main groups in accordance with the subdivision of the family into three genera, *Geodermatophilus*, *Blastococcus* and *Modestobacter*.

Similar results showing a great agreement between fluorescent BOX-PCR clustering and ARDRA and 16S ~~rDNA~~ rRNA gene sequencing have been recently reported for *Modestobacter* and Modestobacter strains isolated from altered carbonatic wall (~~Brussetti~~Brusetti et al., ~~2008~~in ~~press~~2008).

Esterase produced by member of *Geodermatophilus* genus as compared to that of *Blastococcus* and *Modestobacter* genera display a great tolerance to high temperature that rich up to 100°C, enzyme kept 30% of its maximal activity after heating for 120°C.

Such thermal stability is required for industrial application. Enzymes from extremophiles and thermophiles in particular are promising in this respect because these enzymes have a high intrinsic thermal and chemical stability (Levisson., 2007). *Bacillus stearothermophilus* (Owusu et Cowan, 1991; Shao et Wiegel, 1995), *Sulfolobus shibatae* (Huddleston et al., 1995), the hyperthermophilic archaea *Archaeoglobus fulgidus* (Manco et al., 2000), and *Thermotoga maritima* (Levisson et al., 2007) have been shown to contain thermostable esterases.

Under the effect of certain divalent cations, the produced esterase have been activated strongly in presence of $Ca^{2+}$ and $Mg^{2+}$ ions, moderately by $Zn^{2+}$ ions and markedly inhibited by $Cu^{2+}$ and $Co^{2+}$ ions. The purified esterase from *Arthrobacter nicotinea* is inhibited by $Ca^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ and is completely inactivated by $Mg^{2+}$ (Smacchi et al., 2000). It has also been reported that $Ca^{2+}$ and $Mg^{2+}$ ions had no effect on esterase activity purified from *Brevibacterium linens* ATCC9174 (Rattray ~~et~~ and Fox., 1997), however they increased the activity of esterase from *Lactobacillus plantarum* (Gobbetti et al., 1997).

Activation of *Geodermatophilaceae* ~~produced~~ esterase production in presence of $Ca^{2+}$ and $Mg^{2+}$ ions, tolerance to alkaline pH as well as the thermostability observed among *Geodermatophilus* members could be directly linked to the nature of the ecological niche hosted by these actinobacteria and also to the type of the colonized materials~~,~~ ; in fact, it has been reported that alkaline pH as well as porosity linked to the stone itself or to its deterioration status could explain the prevalence of *Geodermatophilaceae* in monuments and stone surfaces with marble and calcareous substrates such as ~~(~~carbonate stones~~),~~ (~~Urzi~~ Urzì et al., 2001).

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According to this data, it can be concluded that stone and monument surfaces provide an ecological complex habitats suitable for the proliferation of diverse range of actinobacteria that show a great similarity in ecotype but seems larger different in genotype and phenotype.

Otherwise, the biodiversity of the rock-inhabiting flora as compared to that of soils microflora is thus relatively high and must be considered different from the ones found in the same ecosystem (Eppard et al., 1996).~~ ~~ ~~Esterase electrophoretic polymorphism proved its efficiency for the differentiation and study of bacterial phylogenetic relationships and served as complementary taxonomic approach to highlight population genetics diversity and phylogeny. ~~The noted flexibility of esterase activities under higher temperature, alkaline pH and higher cationic concentration could be a fitting adaptation of these actinobacteria to such extreme environment represented by calcareous and limestone substrate under arid conditions.

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Table1: Sampling locations and ~~Characterisation~~ genotypic characterisation of ~~actinobacterial~~ Geodermatophilaceae isolates

| Strains | Sample | Location | Country ~~Origin of isolation~~ | Restriction enzymes patterns | | | ARDRA types | Esterase |
|---|---|---|---|---|---|---|---|---|
| | | | | *Msp*I | *Rsa*I | *Cfo*I | | |
| **NRRLB-3579** | Soil | Westgard Pass, California | USA | | | | | |
| ~~**NRRLB-3579**~~ | | | ~~Soil, Westgard Pass, California, USA~~ | M2 | R2 | C4 | 36 | E3 |
| **NRRLB-3577=G20** | soil, Amargosa Desert, Nevada, USA | | ~~soil, Amargosa Desert, Nevada, USA~~ | M1 | R1 | C6 | 37 | E3 |
| **NRRLB-3580** | soil, Zion National Park, Utah, USA | | ~~soil, Zion National Park, Utah, USA~~ | M1 | R1 | C6 | 37 | E4 |
| **NRRL B-24246** | Globigerine limestone, Church Sta Marija Ta'Cwerra, Siggiewi, Malta | | ~~Globigerine limestone, Church Sta Marija Ta'Cwerra, Siggiewi, Malta~~ | M1 | R1 | C6 | 37 | E3 |
| **DD13** | Calcarenite ancient wall Cagliari italy | | ~~Calcarenite ancient wall Cagliari italy~~ | M2 | R2 | C6 | 35 | E3 |
| **BC501** | Carrara marble Italy | | ~~Carrara marble Italy~~ | M20 | R19 | C18 | 38 | E3 |
| **DS17** | Calcarenite ancient wall Cagliari italy | | ~~Calcarenite ancient wall Cagliari italy~~ | M2 | R2 | C6 | 39 | E4 |
| **DS13** | Calcarenite ancient wall Cagliari italy | | ~~Calcarenite ancient wall Cagliari italy~~ | M2 | R2 | C6 | 39 | E2 |
| **DS10** | Calcarenite ancient wall Cagliari italy | | ~~Calcarenite ancient wall Cagliari italy~~ | M2 | R2 | C6 | 39 | NI |
| **BC499** | Carrara marble Italy | | ~~Carrara marble Italy~~ | M2 | R3 | C6 | 39 | E4 |
| BMG5717 | Sandstone Bulla Regia monument (Tunisia) | | ~~Sandstone Bulla Regia monument (Tunisia)~~ | M12 | R20 | C4 | 1 | E8 |
| BMG5725 | Limestone desert (Tunisia) | | ~~Limestone desert (Tunisia)~~ | M12 | R20 | C1 | 2 | E2 |
| BMG5719 | Sandstone Bulla Regia monument (Tunisia) | | ~~Sandstone Bulla Regia monument (Tunisia)~~ | M19 | R21 | C2 | 3 | NI |
| BMG5740 | Marble Bulla Regia monument (Tunisia) | | ~~Marble Bulla Regia monument (Tunsia)~~ | M3 | R4 | C5 | 4 | E2 |
| BMG5748 | Limestone Worker Tomb monument (Egypt) | | ~~Limestone Worker Tomb monument (Egypt)~~ | M3 | R4 | C5 | 4 | E2 |
| BMG5747 | Limestone Worker Tomb monument (Egypt) | | ~~Limestone Worker Tomb monument (Egypt)~~ | M3 | R4 | C5 | 4 | E1 |
| BMG5746 | Limestone Worker Tomb monument (Egypt) | | ~~Limestone Worker Tomb monument (Egypt)~~ | M3 | R4 | C5 | 4 | E1 |
| BMG5745 | Limestone Worker Tomb monument (Egypt) | | ~~Limestone Worker Tomb monument (Egypt)~~ | M3 | R4 | C5 | 4 | E1 |
| BMG5751 | Limestone Mankara monument (Egypt) | | ~~Limestone Mankara monument (Egypt)~~ | M10 | R4 | C5 | 5 | E2 |
| BMG5744 | Limestone Worker Tomb monument | | ~~Limestone Worker Tomb monument~~ | M10 | R4 | C5 | 5 | E2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | (Egypt) | ~~(Egypt)~~ | | | | | |
| BMG5743 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunsia)~~ | M3 | R17 | C5 | 6 | E1 |
| BMG5750 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunsia)~~ | M10 | R18 | C5 | 7 | E9 |
| BMG5739 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunsia)~~ | M10 | R18 | C5 | 7 | E9 |
| BMG5742 | Sandstne Bulla Regia monument (Tunisia) | ~~Sandstne Bulla Regia monument (Tunsia)~~ | M11 | R18 | C5 | 8 | E4 |
| BMG5738 | Marble desert (Tunisia) | ~~Marble desert (Tunsia)~~ | M3 | R18 | C5 | 9 | E2 |
| BMG5732 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunsia)~~ | M4 | R7 | C12 | 10 | E1 |
| BMG5734 | Sandstone Desert (Tunisia) | ~~Sandstone Desert (Tunisia)~~ | M4 | R7 | C12 | 10 | NI |
| BMG5759 | Sandstone Bulla Regia monument (Tunisia) | ~~Sandstone Bulla Regia monument (Tunsia)~~ | M16 | R8 | C9 | 11 | E1 |
| BMG5757 | Limestone Bulla Regia monument (Tunisia) | ~~Limestone Bulla Regia monument (Tunsia)~~ | M18 | R19 | C16 | 12 | E |
| BMG5728 | Sandstone Desert (Tunisia) | ~~Sandstone Desert (Tunisia)~~ | M17 | R14 | C17 | 13 | E1 |
| BMG5760 | Sandstone Bulla Regia monument (Tunisia) | ~~Sandstone Bulla Regia monument (Tunsia)~~ | M6 | R6 | C13 | 14 | E |
| BMG5735 | Sandstone Desert (Tunisia) | ~~Sandstone Desert (Tunsia)~~ | M5 | R6 | C11 | 15 | E2 |
| BMG5727 | Sandstone Desert (Tunisia | ~~Sandstone Desert (Tunsia~~ | M5 | R6 | C10 | 16 | E1 |
| BMG5713 | Limestone Desert (Tunisia) | ~~Limestone Desert (Tunisia)~~ | M12 | R15 | C4 | 17 | E1 |
| BMG5730 | Limestone Bulla Regia monument (Tunisia) | ~~Limestone Bulla Regia monument (Tunisia)~~ | M8 | R3 | C14 | 18 | E1 |
| BMG5733 | Sandstone Bulla Regia monument (Tunisia) | ~~Sandstone Bulla Regia monument (Tunisia)~~ | M7 | R6 | C4 | 19 | E1 |
| BMG5723 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M12 | R5 | C4 | 20 | E1 |
| BMG5722 | Limestone | ~~Limestone~~ | M13 | R5 | C4 | 21 | E1 |
| BMG5718 | Limestone Tomb monument (Egypt) | ~~Limestone Tomb monument (Egypt)~~ | M1 | R5 | C4 | 22 | E1 |
| BMG5716 | Limestone Desert (Tunisia) | ~~Limestone Desert (Tunisia)~~ | M13 | R15 | C2 | 23 | E1 |
| BMG5720 | Sandstone Desert (Tunisia) | ~~Sandstone Desert (Tunisia)~~ | M13 | R5 | C2 | 24 | NI |
| BMG577 | Limestone Tomb monument (Egypt) | ~~Limestone Tomb monument (Egypt)~~ | M12 | R5 | C2 | 25 | E1 |
| BMG571 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M1 | R2 | C15 | 26 | E2 |
| BMG575 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M1 | R2 | C3 | 27 | E2 |
| BMG572 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M1 | R2 | C3 | 27 | E2 |
| BMG5712 | Limestone Desert | ~~Limestone Desert~~ | M1 | R1 | C3 | 28 | NI |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | (Tunisia) | ~~(Tunisia)~~ | | | | | |
| BMG576 | Limestone Tomb monument (Egypt) | ~~Limestone Tomb monument (Egypt)~~ | M1 | R1 | C3 | 28 | E2 |
| BMG5714 | Marble Desert (Tunisia) | ~~Marble Desert (Tunisia)~~ | M1 | R1 | C3 | 28 | E3 |
| BMG574 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M1 | R1 | C3 | 28 | E3 |
| BMG573 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M1 | R1 | C3 | 28 | E2 |
| BMG5711 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M1 | R1 | C3 | 28 | E2 |
| BMG5710 | Limestone Bulla Regia monument (Tunisia) | ~~Limestone Bulla Regia monument (Tunisia)~~ | M1 | R1 | C3 | 28 | E3 |
| BMG5715 | Limestone Desert (Tunisia) | ~~Limestone Desert (Tunisia)~~ | M1 | R2 | C1 | 29 | E2 |
| BMG579 | Marble Bulla Regia (Tunisia) | ~~Marble Bulla Regia (Tunisia)~~ | M1 | R1 | C1 | 30 | E2 |
| BMG578 | Limestone Tomb monument (Egypt) | ~~Limestone Tomb monument (Egypt)~~ | M1 | R1 | C1 | 30 | E2 |
| BMG5729 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M2 | R13 | C3 | 31 | E3 |
| BMG5753 | Limestone Bulla Regia monument (Tunisia) | ~~Limestone Bulla Regia monument (Tunisia)~~ | M2 | R9 | C7 | 32 | NI |
| BMG5754 | Limestone Tome monument (Egypt) | ~~Limestone Tome monument (Egypt)~~ | M2 | R11 | C7 | 33 | NI |
| BMG5756 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M15 | R12 | C8 | 34 | NI |
| BMG5755 | Limestone Bulla Regia monument (Tunisia) | ~~Limestone Bulla Regia monument (Tunisia)~~ | M2 | R10 | C6 | 40 | NI |
| BMG5726 | Marble Bulla Regia monument (Tunisia) | ~~Marble Bulla Regia monument (Tunisia)~~ | M2 | R3 | C6 | 41 | E4 |
| BMG5731 | Sandstone Bulla Regia monument (Tunisia) | ~~Sandstone Bulla Regia monument (Tunisia)~~ | M2 | R3 | C6 | 41 | E4 |
| BMG5758 | Sandstone Bulla Regia monument (Tunisia) | ~~Sandstone Bulla Regia monument (Tunisia)~~ | M2 | R3 | C6 | 41 | E4 |
| BMG5724 | Limestone Khufu pyramid (Egypt) | ~~Limestone Khufu pyramid (Egypt)~~ | M14 | R3 | C6 | 42 | E3 |
| BMG5721 | Limestone khafre pyramid (Egypt) | ~~Limestone khafre pyramid (Egypt)~~ | M1 | R3 | C6 | 43 | NI |
| BMG5749 | Limestone Worker Tomb monument (Egypt) | ~~Limestone Worker Tomb monument (Egypt)~~ | M9 | R16 | C6 | 44 | E4 |
| BMG5737 | Sandstone Desert (Tunisia) | ~~Sandstone Desert (Tunisia)~~ | M9 | R3 | C6 | 45 | E4 |

1 ND: not determined

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**Figure comments**

**Fig.1.**~~–~~ Phylogenetic tree based on 1307 bp of the 16S rRNA gene obtained by neighbor-joining algorithm. Nucleotide sequence accession numbers are indicated in parentheses. Bootstrap values determined from 1000 replicates are indicated.

**Fig.2.** Examples of esterase patterns of ~~actinobacterial~~ Geodermatophilaceae strains resolved on 12% native polyacrylamide gel electrophoresis.

**Fig.3.** Phylogenetic relationship between Geodermatophilaceae ~~actinobacterial~~ isolates based on esterase patterns. The dendrogram was generated by UPGMA/Jaccard coefficient cluster analysis of esterase band.

**Fig.4.** Temperature effect on ~~crude esterase~~ activity of crude esterases; A: esterases from *Blastococcus*, B: esterases from *Modestobacter*,~~from Geodermatophilaceae~~ C: esterases from *Geodermatophilus*.

**Fig.5.** pH effect on activity of crude esterases; A: esterases from *Blastococcus*, B: esterases from *Modestobacter*, C: esterases from *Geodermatophilus*.

~~crude esterase activity from *Geodermatophilaceae*.~~