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Case study

Carbonatogenic bacteria on the 'Motya Charioteer' sculpture

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ABSTRACT

The 'Motya Charioteer' marble statue, a masterpiece of ancient Greek sculpting, was discovered in 1979 on the island of Motya, Sicily. A general assessment of the statue's conditions in 2008 revealed that the marble has lost its luster and started to show some microcracks. In 2019 and in 2020, microbiological surveys were conducted to assess the marble's biodeterioration through the identification of bacteria capable of metabolizing calcium carbonate and damaging the statue. Bacterial strains with calcium carbonate dissolution properties were isolated exclusively from the damaged areas of the statue; among 31 strains showing calcium carbonate metabolism (precipitation and/or dissolution), 23 were bacilli. While causation cannot be confirmed, these bacterial strains are certainly capable of dissolving marble leading to statue degradation. In two damaged areas of the statue, *Staphylococcus haemolyticus*, a common component of human skin flora was identified. This strain demonstrated a fast calcium carbonate dissolution property, which has not previously reported for this species. Finally, two strains (*Lysinibacillus fusiformis* and *Metabacillus litoralis*), showed carbonatogenic features perfectly suitable for a bioconsolidation intervention on the sculpture.

1. Introduction

The'Motya Charioteer' statue, an ancient Greek sculpture of a young male charioteer, was discovered in 1979 during an archeological excavation on the island of Motya, Sicily. The statue was located in the vicinity of the Sanctuary of 'Cappiddazzu', a monumental temple, devoted to the Phoenician god Melqart, that was in use between the 8th and the 4th century BCE. This statue is a testimony to the wealth of this Phoenician city and the spirit of its ruler and matches in splendor similar icons of the Sicilian Greek cities at the height of their grandeur. The statue, now on exhibit in the G. Whitaker Museum in Motya, was carved in Parian marble (Fig. 1) and, at the time of its discovery, it was lacking its feet and was broken at the level of the malleoli. Its eyes, nose, and mouth were intentionally scarred, most likely mutilated during the siege and destruction of the city by Dionysius of Syracuse in 397 BCE. In 2008, a general condition assessment of the sculpture showed damages that could have been caused by the dire environmental conditions in which the statue was discovered. Its condition was further worsened through spontaneous degradation following the sculpture unearthing, and in the lack of proper precautionary procedures of displacement for international exhibitions.

Some of these degradation factors, either individually or in consort, continue to act today on the statue surface. At the time of discovery, the statue had some parts still immersed in a puddle of water. Parts of the back, buttocks and left calf suffered from being left in a semi-watered environment for an extended period which caused visible damages to its surface in certain spots. The parts fully immersed in water kept the statue in a relatively good state of preservation, while those directly above the line of dive were more damaged showing a decohesion surface with bleaching and loss of crystal structure of the marble. A widespread microcracking in the crystalline layer of the marble was also observed, resulting in a diffuse opaque bleaching with loss of marble shine (Fig. 2).

Monitoring the preservation state of the statue is a standard procedure of the G. Whitaker Museum where the sculpture is exhibited and, recently, a microbiological analysis was performed to investigate the state of biodegradation of the statue. A survey of the statue was conducted with a specific purpose to investigate the presence of bacterial strains that may induce precipitation (carbon-

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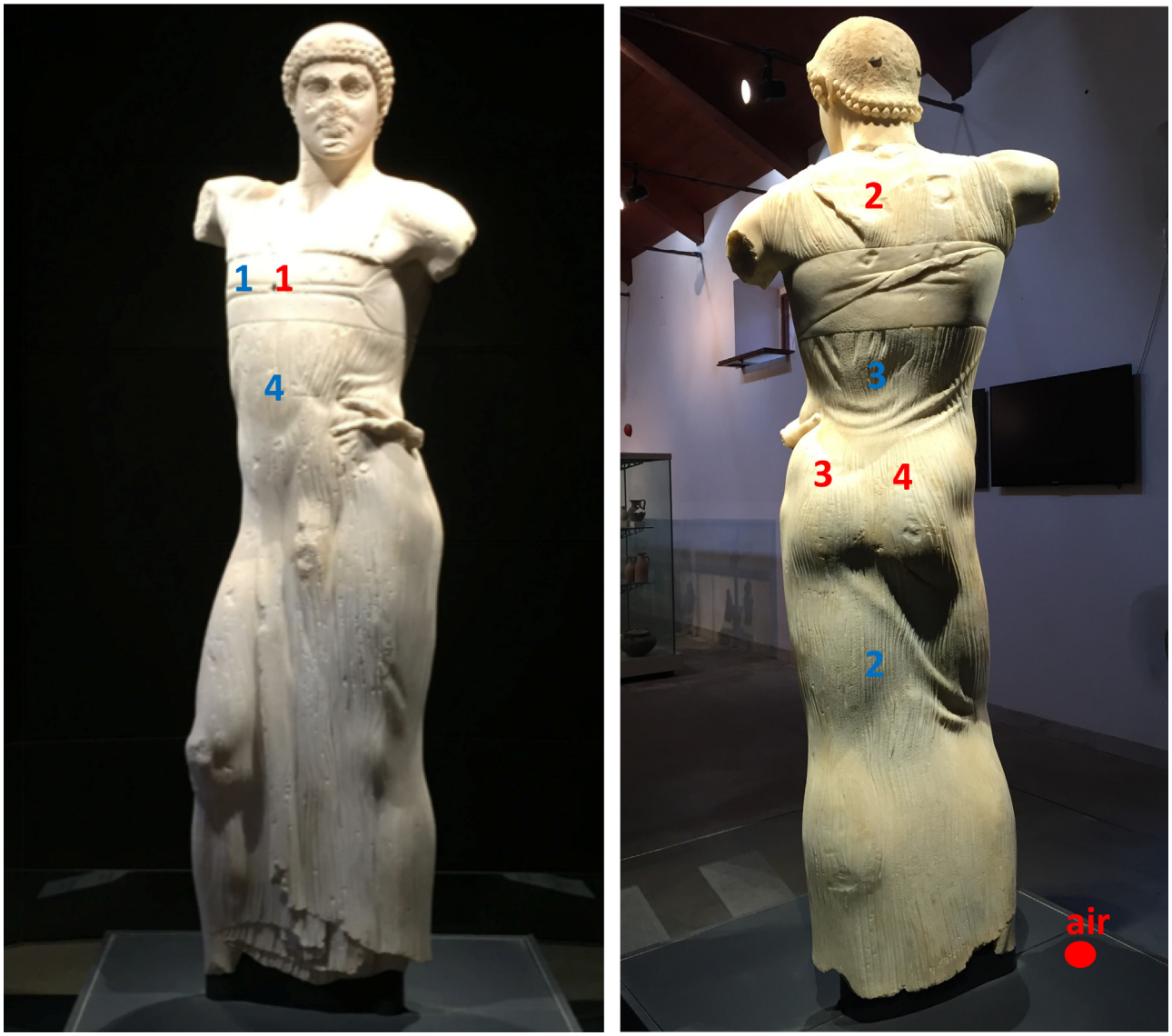

Fig. 1. The 'Motya Charioteer' statue (exhibit in the G. Whitaker Museum in Mozia, Sicily). Numbers indicate the sites where microbiological analyses were performed in 2019 (red numbers) and 2020 (blue numbers). Sites in good preservation state were 1 and 2 (red), and 1 (blue). The damaged areas were marked as 3 and 4 (red) and 2, 3, 4 (blue). The red dot indicates the position where a control plate was left open during the sampling.

atogenic) and/or dissolution of calcium carbonate. The presence of carbonatogenic bacteria for a possible future intervention of bioconsolidation was also investigated.

There is extensive literature describing bacterial involvement in carbonate precipitation [1–4]. One of the many applications of this bacterial process is in the conservation of stone artworks [5–8], taking advantage of the bacterial ability to induce carbonate precipitation [9–11]. The microbially induced calcite precipitation (MICP) is a natural phenomenon among bacterial species promoted by their urease activity in an alkaline environment rich in calcium ions [3,12]. Here, we report the results of the microbiological survey conducted in April 2019 on the 'Motya Charioteer' statue and a second sampling of separate damaged areas of the marble conducted in September 2020.

1.1. Research aim

This study investigates a bacterial community colonizing a Greek sculpture discovered in 1979 during an archeological excavation in a Phoenician site on the island of Motya, Sicily.

The contribution of microbiology to the field of cultural heritage is widespread, but to date it is mostly applied when the biodeterioration of an artifact becomes evident to the naked eye. The aim of this work is to conduct a microbiological survey on a marble sculpture that was not subjected to any evident biological attack. On the sculpture, we also investigated the presence of bacterial strains with a metabolic activity promoting precipitation and/or dissolution of calcium carbonate. The results presented are of a general interest and can be applied in the preservation of marble masterpieces exhibited in museums.

2. Materials and methods

2.1. Media and growth conditions

YPD: 1% Bacto-peptone,1% Yeast Extract, 2% Glucose and 2,1% Agar; LB: 0,5% Yeast Extract, 1% Bacto-Tryptone, 0,5% NaCl, 1 ml NaOH 1 N and 2,1% Agar; B4-C: 0.4% yeast extract, 0.5% glucose, 0.25% $CaCO_3$ and 1.4% agar; YPD+urea+$CaCl_2$: 1% Bacto-peptone, 1% yeast extract, 2% glucose, 3 g/L of urea, 25 g/L of $CaCl_2$ and

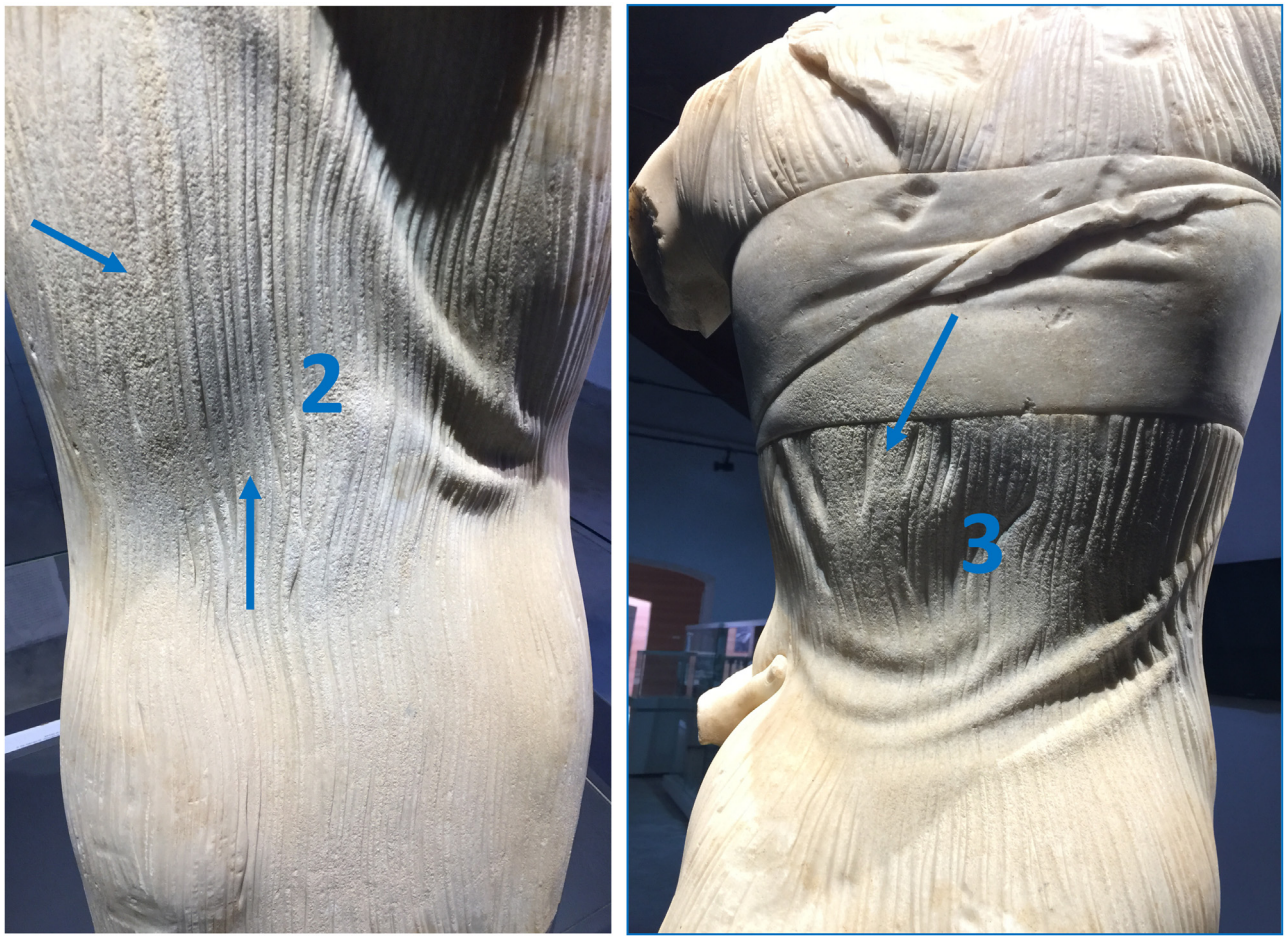

Fig. 2. Phenomenon of decohesion with bleaching and loss of crystal structure (arrows) in the 'Motya Charioteer' marble. The numbers indicate the sites of the 2020 microbiological sampling.

2% agar. Calcium chloride was chosen as a source of calcium ions because promotes calcite crystals deposition, while calcium acetate promotes aragonite production [13]. Strains used for microbiological analysis were streaked in sterile conditions on plates and grown at room temperature (25 °C). The production and the dissolution of calcium carbonate was monitored on YPD+urea+$CaCl_2$ or B4-C plates.

2.2. Isolation and characterization of bacterial strains

The non-invasive sampling procedures were performed by gently pressing sterile velvets (3 cm x 3 cm) onto the sites of the statue, and immediately streaking them on plates. YPD plates were used in the 2019 sampling, and YPD+urea+$CaCl_2$ and B4-C plates in 2020. The standard culture techniques used for the screening, and for further analysis, were restrictive and did not allow the growth of the entire microbial community. During the sampling, a YPD plate remained open as a control for air contamination assessment. After 4 days at room temperature, bacterial colonies grown on plates were observed with an optical microscope and transferred on YPD plates for further analysis (Fig. S1a). The first survey (2019) was conducted on sites 1–4 (Fig. 1, red numbers). To support the results of the first survey, different deteriorated areas of the statue (sites 1–4, Fig. 1, blue numbers) were further investigated with a second sampling conducted in September 2020. In the control plate, left open during the sampling, only a mold was grown (not shown). The isolates were designated with a first number, which indicates the site where the strains were isolated, followed by a progressive number (Table 1). Metagenomic analysis was not performed since the focus was to verify the presence of strains showing a calcium carbonate metabolism and to culture those strains suitable for a bioconsolidation. Molecular characterization of the isolated bacteria was performed by DNA extraction: a single colony for each bacterial strain was picked and suspended in 180μl of Genes Lysis Buffer (BIO-RAD) (50 mM Tris, 1%SDS, pH 8) with 36μl lysozyme (20 mg/ml). Heated at 37 °C for 30 min. Centrifuged at 4 °C for 5 min and suspended in 0.4 mL of AE buffer plus 1% (w/v) SDS (AppliChem GmbH). Cells were lysed with phenol:chloroform (5: 1, pH 4.7, Sigma-Aldrich), heated at 65 °C for 10 min, transferred at −80 °C for 10 min and the aqueous phase was separated by centrifugation. After a second extraction with phenol:chloroform (24: 1, pH 5.2, Sigma-Aldrich), DNA was precipitated with ethanol, dried and suspended in sterile water. DNA quantity and purity were assessed with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) at 260 nm and at 260/230, 260/280 nm ratios, respectively. DNA integrity was assessed by electrophoresis on ethidium bromide stained 1% agarose-formaldehyde gels. The primers F8 (50-AGAGTTTGATCCTGGCTCAG-30) and R1492 (50- GGTTACCTTGTTACGACTT-30) were used to amplify a region of approximately 1100 bp from the 16S rRNA gene. The PCR reaction was performed utilizing Taq DNA polymerase from Accuzyme DNA Polymerase (Bioline). SARA ENViMOB S.r.l. (Rome, Italy) amplified and sequenced 16S rRNA gene using the Sanger method, and the obtained sequences were analyzed

Table 1
Identification of closest bacterial relatives of strains isolated from the 'Motya Charioteer' statue based on 16S rRNA gene sequence and microscopic observation. The isolates were named with a first number, which indicates the site where the strains were isolated, followed by a progressive number. In 2020, two strains, *Staphylococcus haemolyticus* and *Micrococcus luteus*, were selected in two sites, the sites 2 and 3 and 3 and 4, respectively. The strain 4.22 was identified only by microscopic analysis. The carbonatogenic phenotype of the strains was tested on B4-C and YPD+urea+$CaCl_2$ plates. "/" indicates the absence of precipitation and dissolution phenotypes.

Isolates 2019	The closest bacterial relatives	Identity%	Gene bank accession number	Carbonatogenic properties
3.15	*Bacillus nealsonii*	98,912	MZ702666	precipitation and dissolution
3.19	*Peribacillus simplex*	99,547	MZ702667	precipitation and dissolution
3.20	*Lysinibacillus fusiformis*	99,319	MZ702668	precipitation
3.21	*Mesobacillus subterraneus*	93,103	MZ702669	precipitation and dissolution
3.22	*Mesobacillus jeotgali*	99,317	MZ702670	precipitation and dissolution
3.24	*Terribacillus goriensis*	99,82	MZ702671	dissolution
3.34	*Terribacillus saccharophilus*	99,656	MZ702673	precipitation
4.14	*Bacillus zhangzhouensis*	99,494	MZ702680	precipitation
4.18	*Metabacillus litoralis*	99,818	MZ702682	precipitation
4.19	*Streptomyces fimicarius/griseus/parvus*	98,926	MZ702683	precipitation and dissolution
4.22	*Sporosarcina ureae*	–	–	precipitation and dissolution
4.24	*Bacillus haynesii*	93,036	MZ702687	precipitation
4.25	*Brachybacterium tyrofermentans*	99,90	MZ702688	dissolution
4.28	*Bacillus mobilis*	99,671	MZ702689	precipitation
Isolates 2020				
1.2	*Priestia aryabhattai*	99,549	MZ702657	/
1.4	*Bacillus* sp. *Y1*	99,369	MZ702658	/
1.5	*Microbacterium oleivorans*	98,957	MZ702659	/
2.3	*Paenibacillus lautus*	99,816	MZ702660	dissolution
2.4, 2.5, 2.7, 3.6	*Staphylococcus haemolyticus*	100	MZ702661, MZ702662, MZ702664, MZ702676	dissolution
2.6	*Bacillus circulans*	99,406	MZ702663	dissolution
3.1	*Bacillus nealsonii*	99,506	MZ702665	dissolution
3.3, 3.4, 4.6	*Micrococcus luteus*	99,814	MZ702672, MZ702674, MZ702691	precipitation
3.5	*Bacillus benzoevorans*	99,805	MZ702675	dissolution
4.5	*Brevibacterium casei*	97,688	MZ702690	dissolution
4.7	*Bacillus mycoides*	100	MZ702692	precipitation and dissolution
4.9	*Priestia megaterium*	99,809	MZ702693	precipitation
4.10, 4.14	*Bacillus licheniformis*	99,313	MZ702677	precipitation
4.11	*Bacillus paralicheniformis*	99,71	MZ702678	precipitation and dissolution
4.13	*Streptomyces* sp.	99,728	MZ702679	precipitation
4.16	*Peribacillus simplex*	99,582	MZ702681	precipitation
4.20, 4.21, 4.22	*Neobacillus niacini*	99,826	MZ702684, MZ702685, MZ702686	precipitation

with the BLAST database. The bacterial species characterized in this work are reported in Table 1, Genebank accession number: MZ702657- MZ702693.

2.3. Scanning electron microscopy analysis

SEM micrographs were obtained using a Field Emission Scanning Electron Microscopy (FESEM) Zeiss Auriga 405, with a chamber room that maintains a pressure of about 10−5 to 10−6 mbar. Before mounting the samples inside the microscope, the specimens were coated with 20 nm of chromium using a Quorum Q150T sputter. Chromium has a high X-ray Kα value (5.145 keV), and does not interfere with lighter elements during the EDX analysis. EDX spectra were obtained using a Bruker Quantax detector in point mode for 30 s, with the electron microscope acceleration voltage set at 10 kV and working distance of 6 mm to optimize the number of the incoming X-ray signal.

3. Results

3.1. Microbiological analysis of the 'Motya Charioteer' sculpture

A careful inspection of the 'Motya Charioteer' sculpture revealed the presence of damaged areas, in the first survey of 2019, six strains we collected from the air control and from samples 1 and 2. A total of 34 and 38 colonies were respectively recovered from samples 3 and 4 (the deteriorated areas) (Table S1). Some strains did not survive at the growth conditions used in the laboratory (Fig. S1a). To investigate a possible role of the bacterial metabolism in marble deterioration, colonies were tested on B4-C medium, a minimal medium supplemented with calcium carbonate ($CaCO_3$) used to identify strains able to dissolve $CaCO_3$, and on YPD medium supplemented with urea and calcium chloride ($CaCl_2$). In the latter medium, bacteria able to promote $CaCO_2$ deposition using the urea metabolism can be identified [14]. Table S1 summarized the results obtained by testing the ability of bacteria to dissolve or to precipitate calcium carbonate: 7 bacterial strains able to dissolve and/or precipitate calcium carbonate were identified from damaged sites 3 and 4 but not from the areas of marble in good condition or from the air control.

Samples collected in September 2019 were directly grown on B4-C and YPD+urea+$CaCl_2$ plates, to enrich and select bacterial strains with a calcium carbonate metabolism (Fig. 1, blue numbers). Site 1 was considered a control area because the marble was in a perfect state of preservation, while sites 2, 3 and 4 appeared deteriorated (Fig. 2 and Fig. S2). The number of colonies obtained during the 2020 survey are summarized in Table S1. A total of 4, 7, 5, and 18 colonies were selected from sites 1, 2, 3 and 4, respectively. In this second sampling, the lower number of bacterial strains recovered reflected the use of selective sampling media. Of note, also in the second sampling, the culturable bacterial strains with calcium carbonate precipitation and dissolution

phenotypes were recovered only from the deteriorated areas of the statue, three strains from sites 3 and 4, and 7 from site 4.

3.2. Molecular identification of carbonatogenic bacterial strains

To identify the bacterial species present on the statue, 16S rDNA gene sequencing was performed on a selection of strains (see Materials and Methods). From the sampling of 2019, only strains able to precipitate or dissolve calcium carbonate, only retrieved from the degraded areas (sites 3 and 4) were sequenced. The strain 4.22, which can dissolve and precipitate calcium carbonate, was not studied using molecular methods because it was identified as *Sporosarcina ureae* by optical and electron microscopy observations (Fig. S3) because this is the only species with an established *Sporosarcina* cocci-shaped structure [15].

From the 2020 sampling,16S rDNA sequencing was performed on all the culturable strains. Table 1 lists the identified bacterial species isolated from the 'Motya Charioteer' statue based on 16S rRNA gene sequence. Bacilli was predominant; 23 out of 31 strains were *Bacillus, Neobacillus, Peribacillus, Lysinibacillus, Mesobacillus, Terribacillus, Metabacillus, Paenibacillus* and *Priestia* (previously classified as *Bacillus* [16,17]). The remaining genera other than Bacilli were two *Streptomyces* strains, and a single strain for each of the following genera: *Brachybacterium, Brevibacterium, Microbacterium, Micrococcus, Staphylococcus* and *Sporosarcina*.

Bacillus nealsonii was identified from the 2019 survey (strain 3.15 from site 3, red number in Fig. 1) and also in the 2020 survey (strain 3.1 from site 3, blue number in Fig. 1). *Peribacillus simplex* was also identified in 2019 (strain 3.19 from site 3, red number in Fig. 1) and in 2020 (strain 4.16 from site 4, blue number in Fig. 1). Among the sequenced strains, in the sampling survey of 2020, *S. haemolyticus* was retrieved from two degraded sites (2 and 3) of the sculpture; this strain produced a halo around the colonies on B4-C plates, indicating that calcium carbonate was dissolved (Fig. S4). The first colony to appear on the plates, 24 h after the 2020 sampling, was the *Priestia aryabhattai* strain 1.2, (Fig. S5a), and did not show a calcium carbonate precipitation or dissolution phenotype. This sample was selected from site 1, where the marble was in perfect conditions. *P. aryabhattai* strain 1.2 produced large amount of extracellular polymeric secretions (EPS) in YPD and B4-C plates, but not in LB and YPD +urea+$CaCl_2$ plates (Fig. S5b).

Thus, bacterial strains with carbonate dissolution and deposition properties were isolated only from degraded areas of the sculpture, and many of them showed both properties (see Discussion). In sites with good preservation state (1 and 2, 2019 and site 1, 2020, showed in Fig. 1), the number of culturable bacterial strains was lower than the one in the damaged areas; 16 colonies in three control sites compared to 102 colonies in 5 damaged areas (Table S1).

3.3. Biomineralizing activity of Metabacillus litoralis strain 4.18 and Lysinibacillus fusiformis strain 3.20

The use of biomineralization in the field of cultural heritage is an emerging area of interest, and we sought to consider endogenous strains that can precipitate large amount of $CaCO_3$ as possible candidates for marble bioconsolidation. For such a purpose, strains with a high biomineralization activity and low dissolution of $CaCO_3$ are required, thus different combinations of strains on YPD+ urea+$CaCl_2$ plates were tested. After screening of the calcium carbonate precipitated of the carbonatogenic strains by electron microscopy analysis (not shown), two strains were selected, *L. fusiformis* strain 3.20 and *M. litoralis* strain 4.18, for further analysis. At the tested conditions, these strains precipitated large amount of $CaCO_3$ with a very low dissolution activity (see Discussion) (Fig. S6). Samples from the deposition of *L. fusiformis* strain 3.20 and *M. litoralis* strain 4.18 (indicated as stars in Fig. S6) were sampled directly from YPD+urea+$CaCl_2$, and an EDX analysis confirmed that the deposition produced on plates was indeed $CaCO_3$ (Fig. S6). Scanning electron micrographs of the *L. fusiformis* strain 3.20, a motile strain, and *M. litoralis* strain 4.18, a non-motile strain, are shown in Figs. 3 and 4, respectively. Both strains precipitated large crystals of $CaCO_3$ with a spherulitic shapes (Figs. 3a and 4a). Fig. 3d shows the biofilm of *L. fusiformis* strain 3.20 with bacterial cells embedded on the surface of $CaCO_3$ deposition. A biofilm completely covered the surface of the $CaCO_3$ spherulitic shape, shown in the detail in Fig. 3e and f. Fig. 4d shows the calcification of the *M. litoralis* 4.18 cells.

4. Discussion and conclusions

The contribution of microbiology to the field of cultural heritage is widespread, but mostly applied when the biodeterioration of an artifact (paintings, books, leather, parchments, hypogeal mural paintings, historical buildings) becomes evident to the naked eye [18–24]. This work represents the first case of a microbiological survey on a masterpiece, a marble statue without an apparent biological attack. In order to assess a possible role of bacteria in the deterioration of the marble, growth media were used to purposefully select bacterial strains with a metabolic activity promoting precipitation and dissolution of calcium carbonate. The results presented here pointed to a major concern: the sites showing marble decohesion were more bioreceptive than the areas in which the marble was well preserved, and the damaged areas of the statue were enriched in bacteria able to promote calcium carbonate precipitation and/or dissolution. Among those strains, the *Bacillus* genus was overrepresented, and surprisingly, a few bacterial species that are common to the human skin flora, were also isolated. These findings suggest that these strains may contribute to marble decay whenever their metabolism is activated. Hence, to avoid further degradation, it in an absolute requirement to keep environmental conditions (temperature and humidity) of the statue stable to avoid the activation of the bacterial metabolism. This observation is further supported by the fact that the spore forming bacteria strains identified by molecular method (16S rDNA sequence), were predominant. Fortunately, those species were present in a dormant stage without an active metabolism. A careful visual inspection did not reveal any trace of growth such as colonization or biofilm formation on the sculpture surface. The ability to produce spores confers to bacteria high resistance to harsh conditions. The *B. nealsonii* strain, recorded in 2019 and in 2020 on the 'Motya Charioteer' sculpture, was first isolated in a spacecraft-assembly facility that is sterilized routinely. This strain was shown to be resistant to UV, g-radiation, H_2O_2 and desiccation [25].

Among the identified species on the sculpture, *Bacillus licheniformis, Bacillus mobilis, Micrococcus luteus, P. simplex* and *Priestia megaterium,* were previously described as capable to precipitate $CaCO_3$ [13,26,27]. Strains of *B. licheniformis,* L. *fusiformis* and *P. megaterium* were previously isolated from loamy or calcareous soils and described as carbonatogenic strains [27,28]. *Sporosarcina* species are widely studied for their carbonatogenic properties [29–31]. Members of the *Streptomyces* genus are also known to precipitate $CaCO_3$ [10,32].

The presence on the sculpture of bacterial strains able to precipitate and dissolve calcium carbonate was not surprising, since the substrate is marble, and the calcium carbonate metabolism is common among bacteria [33]. However, our results demonstrated that most of the bacterial strains identified were able to depose $CaCO_3$ and subsequently dissolve it, or able to dissolve $CaCO_3$ produced by neighboring strains (an example in Fig. S6). The precipitation and dissolution properties of many strains is of concern be-

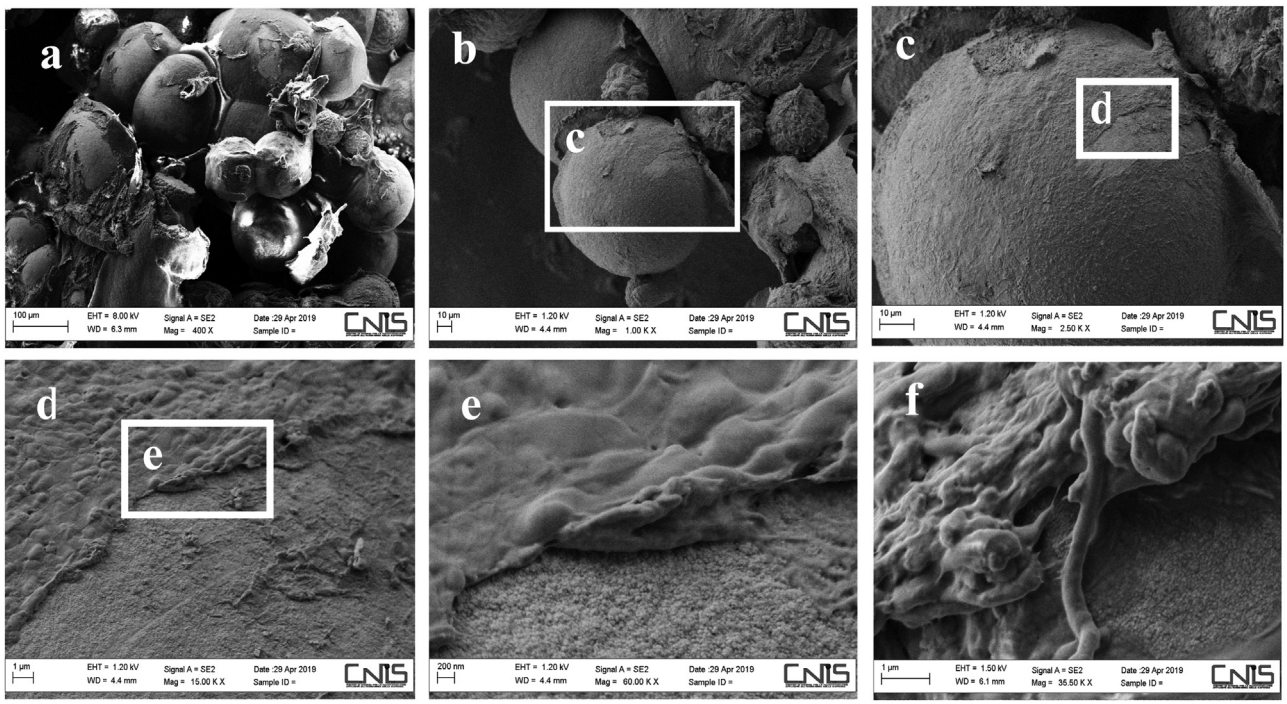

Fig. 3. The *Lysinibacillus fusiformis* 3.20, a motile strain, precipitates spherulitic shaped crystals of $CaCO_3$. a-b: scanning electron micrographs of $CaCO_3$ on plates of YPD + urea + $CaCl_2$ (corresponding to black star in suppl Fig. 4). c, d and e: enlarged view of the insets in b, c, d, respectively. e and f show the $CaCO_3$ crystals covered by bacteria embedded in a biofilm.

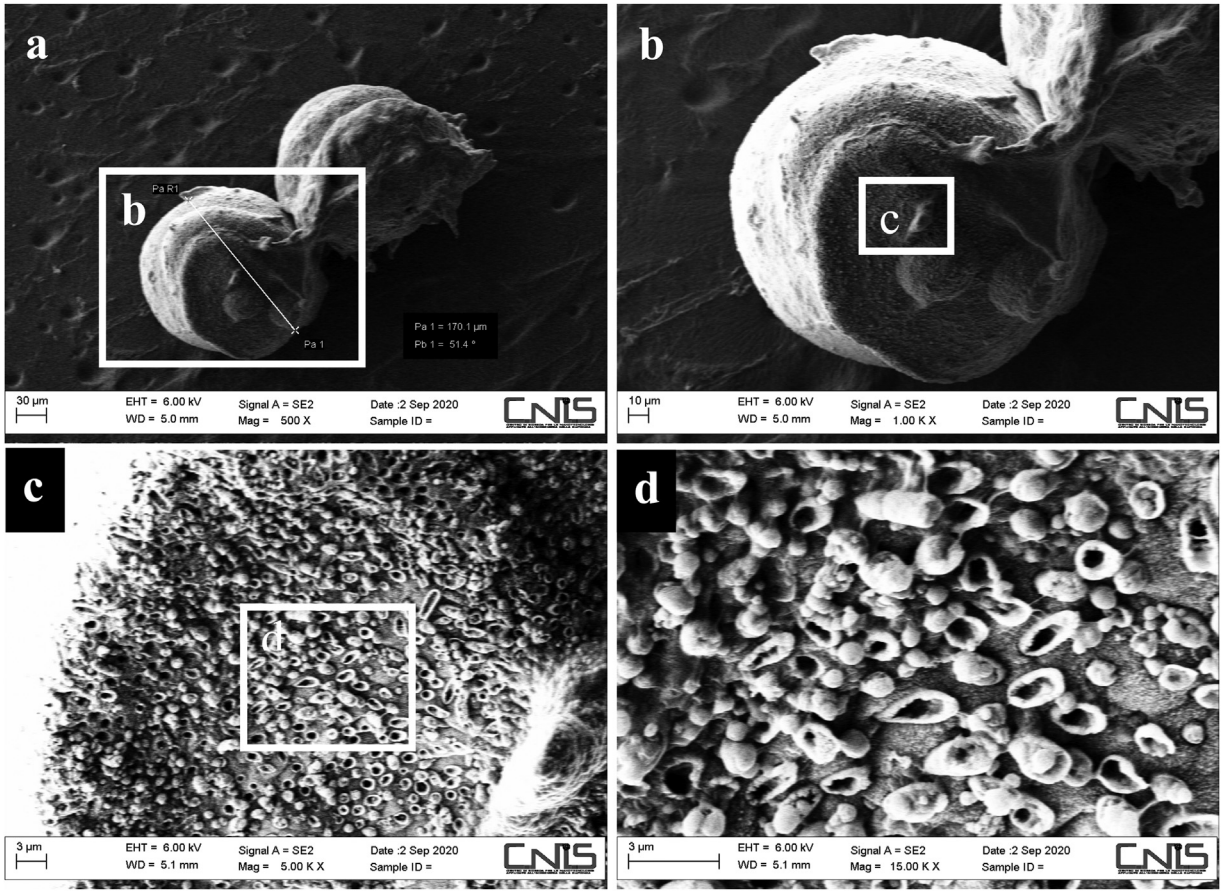

Fig. 4. The *Metabacillus litoralis* strain 4.18 precipitates spherulitic shaped crystals of calcium carbonate. a: scanning electron micrographs of $CaCO_3$ produced on YPD supplemented with urea and $CaCl_2$ (corresponding to white star in suppl Fig 4). The diameter of the $CaCO_3$ in the inset b is 170 mm. b, c and d: enlargements of the inset in a, b and c, respectively, showing bacterial cells calcification.

cause it clearly indicates that any hypothetical addition of a strain for bioconsolidation (even an endogenous strain) could activate the $CaCO_3$ dissolution by the microbial community already present on this artwork.

Two species identified in 2019 and 2020 are commonly associated with milk and cheese: *Brevibacterium casei*, isolated from milk and dairy products [34] and *Brachybacterium tyrofermentans,* first isolated from the surfaces of Gruyère and Beaufort cheeses [35]. Both species are coryneform bacteria, and together with micrococci and staphylococci, constitute a major part of the skin microflora [36,37]. Of note, both strains *B. tyrofermentans* 4.25 and *B. casei* 4.5 showed a $CaCl_2$ dissolution activity on B4-C plates. In the 2020 survey, *S. haemolyticus* was also identified. This species is also commonly found in the human skin microbiota. The presence of *S. haemolyticus* is of concern because it was recovered from the most damaged area of the statue (site 2 and 3, sampling 2020). These strains were able to quickly dissolve calcium carbonate on plates (Fig. S4). It is possible that these strains, often associated with the human microbiota, were left by people who handled the statue in the past. These observations underscore the importance of using protective materials in interventions on cultural heritage objects. The identification of strains that can dissolve $CaCO_3$ in laboratory conditions do not necessarily imply that these bacteria are damaging the statue in situ, but they possess the metabolic activity to do it. Based on these results, the statue is protected and cannot be touched intentionally by tourists. It is also placed in stable environment controlled for temperature and humidity.

Interestingly, in the 2020 survey from site 1, where the marble was in perfect condition, none of three strains identified was able to precipitate or dissolve calcium carbonate at the tested conditions (Table 1): *Microbacterium oleivorans* was previously described as a uranium-tolerant actinobacteria [38] and a hydrocarbon degrading bacterium [39], *Bacillus* sp. Y1 is known for its degumming properties on plant fibers [40], *P. aryabhattai* strains were described to produce EPS and promoting plant growth [41,42]. Indeed, *P. aryabhattai* strain 1.2 showed a peculiar phenotype, it was the first colony to appear on plates 24 h after sampling (Fig. S5a), and it produced large amounts of extracellular polymeric secretions (EPS) only in YPD and B4-C plates (Fig. S5b).

These results underscore the metabolic potentiality of these newly isolated strains and deserve more investigation in the future. Microbial induced calcium carbonate precipitation has potentiality in a variety of fields: atmospheric CO_2 fixation, capturing of inorganic contaminants such as heavy metals, consolidation of limestone, self-healing of concrete, and stabilization of soils [1,4,43,44]. The precipitation of carbonates by bacteria through urea hydrolysis is the most straight- forward and easily controlled mechanism, since it produces high amounts of carbonates in an alkaline environment [5,45]. Bacterial carbonate precipitation was also proposed for enhancing the durability properties of ornamental stone and for the restoration of monuments [46–50]. Indeed, carbonate stones, like limestone and marble, because of weathering, experience a progressive dissolution of the mineral matrix with an increase of porosity and a decrease of mechanical features [50]. Previously, to contrast this decay, many conservation treatments have been applied, such as water repellents or stone consolidants, but unfortunately these types of intervention had often accelerated stone decay [51]. With the urgent need of new eco-friendly solutions, bacterially induced carbonate precipitation was proposed as an alternative, but the large-scale use of microbial calcification has not been always encouraged since it may be hard to manage [5] and the cost of media required for bacterial growth may be a deterrent [52].

Moreover, in the field of cultural heritage, the use of exogenous bacteria remains a concern and the use of indigenous calcifying bacteria for re-inoculation of stone monuments is considered as an alternative strategy for bioconsolidation, initially proposed by Jiménez-López, et al. [47], and with successful results applied by Jroundi et al. [53]. Currently, bioconsolidation using carbonatogenic bacteria was only applied to consolidate decayed building structures and deteriorated ornamental stones and not to sculpture masterpieces. Thus, we sought to conduct a preliminary evaluation of the endogenous carbonatogenic bacteria present on the sculpture to select suitable strains for bioconsolidation if, in the future, this application is to be considered safe for the artifacts and for the environment. Two bacillus strains, *L. fusiformis* strain 3.20 and *M. litoralis* strain 4.18, were considered the most suitable for future studies because in laboratory conditions they promoted the precipitation of calcium carbonate within 24 h, with a low dissolution rate, and produced large spherulitic crystals (Fig. 3 and 4).

While *L. fusiformis* strains were already described in carbonatogenic studies [45,54,55], so far, no strains of *M. litoralis*, first isolated from the sea water [56], were investigated for $CaCO_3$ metabolic activity. In particular, *L. fusiformis* strain 3.20 showed all key properties for carbonate crystallization. It is spore-forming, allowing bacteria to survive in harsh conditions; it is motile, conferring an advantage to deeply colonize the substrate [57]; and it produces EPS, which influences positively $CaCO_3$ precipitation because covering of the surface, plays a role as a nucleation site for $CaCO_3$ precipitation, and calcium carbonate retention, resulting in a homogeneous layer of $CaCO_3$ deposition [14,45,58,59].

To apply carbonatogenic bacteria in bioconsolidation, it is essential to induce precipitation without dissolution of $CaCO_3$, thus, many aspects which influence the equilibrium of precipitation and dissolution, should be considered. The choice of culture medium composition is fundamental to controlling the dynamic process of bacterial biomineralization. Based on the availability of urea or other organic molecules, the equilibrium moves towards dissolution rather than deposition [60]. The calcium ions concentration is also an important parameter. In laboratory conditions, *Brevibacterium linens* strain BS258 induced precipitation or dissolution of $CaCO_3$ while depending on calcium ions concentration [61]. The formation of different anhydrous polymorphs of calcium carbonate (calcite, vaterite and aragonite) is influenced by growth medium: calcium chloride promotes calcite precipitation, while calcium acetate promotes aragonite precipitation [13]. Finally, the choice of the strain is also fundamental, as an example, the *Sporosarcina ureae* strain 4.22, isolated during the 2019 survey, showed a prevalence of dissolution and a low rate of $CaCO_3$ precipitation (Fig. S3), thus it is not suitable for biomineralization applications, at least at the tested conditions.

The overall results obtained discourage the use of these strains even for micro interventions of bioconsolidation on this masterpiece, because many spore-forming strains are present on the statue and their metabolism could be activated accidentally, promoting further damage to the marble. Moreover, only a small fraction of microbial community was studied since this was a classical microbiological survey and several other microorganisms are certainly present on the marble. Many strains did not survive subsequent plate streaking, suggesting that the metabolism of such microbial communities is yet to be fully explored. Nevertheless, the carbonatogenic strains *L. fusiformis* 3.20 strain and *M. litoralis* 4.18, two strains with high $CaCO_3$ precipitation property, could be investigated for other applications, such as soil stabilization, self-healing concrete, monumental stone reinforcement [14,62,63,64] and finally, calcium carbonate dissolving bacteria could be considered in bio cleaning applications [65].

Author contributions

L.N. and T.R. collected samples; A.C., F.M. and T.R. performed the experiments; L.N. and M.P.T. shared the information concerning the sculpture; T.R. conceived the study and wrote the paper.

Additional information

Supplementary information accompanies this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.culher.2022.09.009.

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