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Arthrobacter agilis and rosy discoloration in "*Terme del Foro*" (Pompeii, Italy)

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ABSTRACT

Pink patinas and rosy discolorations on stones and mural paintings are often of biological origin, although they are frequently ascribed to chemical causes. This work represents the first report of this kind of biodeterioration in Pompeii, where we found a pink dusty patina in the "*Terme del Foro*". Non-invasive sampling was performed to identify the aetiology of the discoloration. Interestingly, the pink pigmented bacterial species *Arthrobacter agilis* was found associated to the patina, pointing to a role of this bacterial species in rosy discoloration. Both morphology of the isolate and 16S rDNA analysis were consistent with identification as *A. agilis*. Raman spectroscopy was carried out to compare the pigments from the pink patina with those associated with *A. agilis* isolates, and the reference strain *A. agilis* DSM 20550. A spectroscopically identical carotenoid-like pigment was identified in all the three types of sample, arguing for a biological origin of the patina. Observations of pink patina distribution suggest that low-lighting and salinity are likely to play a role in patina development.

1. Introduction

Pompeii is one of the most important archaeological sites in the world. Despite its importance, Pompeii suffers from a variety of conservation problems. Its deterioration is caused by various physical and chemical processes, such as erosions due to rainfalls and winds, rising damp and saline efflorescence (Maguregui et al., 2012a; Wollner, 2013), as well as by biological agents (Veneranda et al., 2017). Pompeii is included in the list of UNESCO World Heritage, and a plan of restoration has started in 2012, named "Major Project Pompeii", under the sponsorship of the European Community (European Community Decision no. C (2012) 2154, March 29, 2012). Despite the importance of microorganisms in stone deterioration, few data are available on microbial deterioration processes occurring in Pompeii (Caneva et al., 2008; Maguregui et al., 2012b; Veneranda et al., 2017). Growth of fungi, algae and bacteria can cause biodeterioration through biochemical processes such as corrosion, dissolution and solubilization of material, and through biofilm formation and pigmentation on the surfaces (Caneva et al., 2009). Change in coloration of walls and mural paintings is a complex phenomenon, which can arise from chemical or biological processes, or a combination of them (Aze et al., 2006). Confusion arises when the coloration is not evidently due to the presence of pigmented biofilms, or when the discoloration phenomenon does not show the typical epilithic layers, but an intergranular growth of the microorganisms on and inside the stone (Urzì and Realini, 1998; Walker and Pace, 2007). Pink coloured patinas and rosy discolorations are a debated phenomenon, geographically spread in different climatic conditions, and with an apparently scattered distribution, which is now often considered of biological origin. The most frequently reported bacteria associated with pink patinas belong to genus *Rubrobacter* (Gurtner et al., 2000; Schabereiter-Gurtner et al., 2001; Ripka et al., 2006; Imperi et al., 2007; Laiz et al., 2009; Jurado et al., 2012; Kusumi et al., 2013; Ettenauer et al., 2014). Interestingly, not only bacteria were found associated to pink patinas, but also Archaea belonging to the *Halococcus*, *Halobacterium* and *Haloferax* genera (Rölleke et al., 1998; Imperi et al., 2007; Piñar et al., 2009; Ettenauer et al., 2014). Fungi were also identified among the red pigment-producing microorganisms, especially those belonging to the genus *Rhodotorula* (Rosado et al., 2014).

Information on microbial agents contributing to the formation of pink-coloured patinas and rosy discolorations, as well as the geographical distribution and the ecological conditions that favour the development of such microorganisms, would guide in prevention and control of such phenomena. This information is vital when degradation phenomena occur in monuments of international cultural value, where conservative activity is mandatory, and where biodeterioration studies are still poor.

Here we describe for the first time, the presence of rosy discoloration in Pompeii, where a pink dusty patina was identified in the thermal baths "*Terme del Foro*". The main aim of this work was the search for

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and the characterization of microorganism(s) responsible for this type of biodeterioration, using non-invasive sampling procedures.

2. Materials and methods

2.1. Sampling site

The ancient Roman town of Pompeii (Italy, lat: 40°44.7444′ N; long: 14°29.8188′ E) was destroyed in the 79 C.E. by the eruption of Mount Vesuvius. Burned and buried under meters of volcanic ashes and pumice, the city was rediscovered in 1748 by R.J. Alcubierre, a military engineer, and his assistant K.J. Weber, under the auspices of king Charles III of Spain, and then the excavations of the different regions of the city has begun (Özgenel, 2008). In the second half of XIX century, the Italian archaeologist G. Fiorelli divided the city in *regiones* and *insulae*, starting a systematic archaeological excavation. From its rediscovery to modern days, Pompeii has been a popular tourist destination. The ancient town was part of the Gran Tour and today is one of the most popular archaeologic sites in Italy, with more than 3 million visitors per year (http://www.beniculturali.it/mibac/export/MiBAC/index.html). Pompeii is a typical Roman city built along two couples of main streets, two *Cardines* and two *Decumani* that cross each other in perpendicular ways. As others Roman cities, Pompeii has many public buildings such as *Fori*, Temples, theatres and thermal baths, including the "*Terme del Foro*", placed in *insula* no. 5, near the forum of Pompeii, in the VII *regio*.

In this study, we focused our attention on two rooms (the *Tepidarium* and the *Calidarium)* of the male part of the ancient thermal bath "*Terme del Foro*", where a large pink discoloration was identified (Fig. 1). These rooms are semi-confined areas, with gratings separating the inside from the outside. The skylights displayed on roofs let low sunlight enter in both. *Tepidarium* is a rectangular room with a skylight on the top of the ceiling South-South-East (SSE) side. The pink patinas were present in an area comprised between 1.80 and 2.20 m from the floor, on the "atlantes", and in the niches between the sculpted supports in the SSE and South-South-West (SSW) facing walls. Sunlight from the skylight hits these walls in the morning (Fig. 2). *Calidarium* is a rectangular room with an apse in the SSE side (Fig. 1). In *Calidarium* there are four different skylights in the roof, in the South part of the room: one circular in centre of the apse and three square ones (Fig. 1). The patinas are present in an area comprised between 0.4 and 1.0 m from the floor, on frescoes located on the SSW facing wall. The sunlight hits the patinas in early afternoon (Fig. 1).

2.2. Sampling, isolation and cultivation analysis

Samples were collected in November 2015, when outdoor mean temperature was 13,3 °C (http://centrofunzionale.regione.campania.it). The samples were taken from pink dusty patinas (Fig. 1A) located in three different sampling points in *Tepidarium* (A; B; C), and two points in *Calidarium* (D; E) (Figs. 1B and 2). Since the local authority did not allow scraping of surface material, non-invasive sampling was performed with sterile swabs directly rubbed on pink patinas, and immediately used as inocula. Each swab was streaked on two different media: Tryptic Soy Broth Agar (TSA) and TSA supplemented with NaCl (3%, w/v) and $MgSO_4{\cdot}7H_2O$ (2%, w/v) (TSA Na-Mg) to allow the growth of halophilic microorganisms (Jurado et al., 2012). Then, plates were stored aerobically at room temperature for 30 days, while swabs were stored in sterile plastic tubes at 4 °C.

Pink pigmented colonies, that are supposed to be responsible for the patinas, were subcultured on TSA plates and incubated at 37 °C, 26 °C, and room temperature. To check whether pigmentation was influenced by the presence of light, duplicate plates were incubated under both dark and light (Gavrish et al., 2004).

Salt-tolerance tests were performed at 22 °C in both TSB and TSA media supplemented with different NaCl concentrations, expressed as % w/v and Osm/l, as indicated.

2.3. DNA extraction, amplification and sequencing of the 16S gene

Genomic DNA was extracted from 3 ml of pure liquid cultures grown in TSB medium, at 22 °C for 48 h using QIA amp DNA Mini Kit (no. 51306; Qiagen) following the protocol for Gram positive bacteria. The 16S rDNA was amplified by Polymerase Chain Reaction (PCR) using conserved bacteria-specific primers: 27f (forward) and 1492r (reverse) (Lane, 1991). The PCR was performed according to the manufacturer's recommendations (Promega). PCR thermal conditions used were: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s,

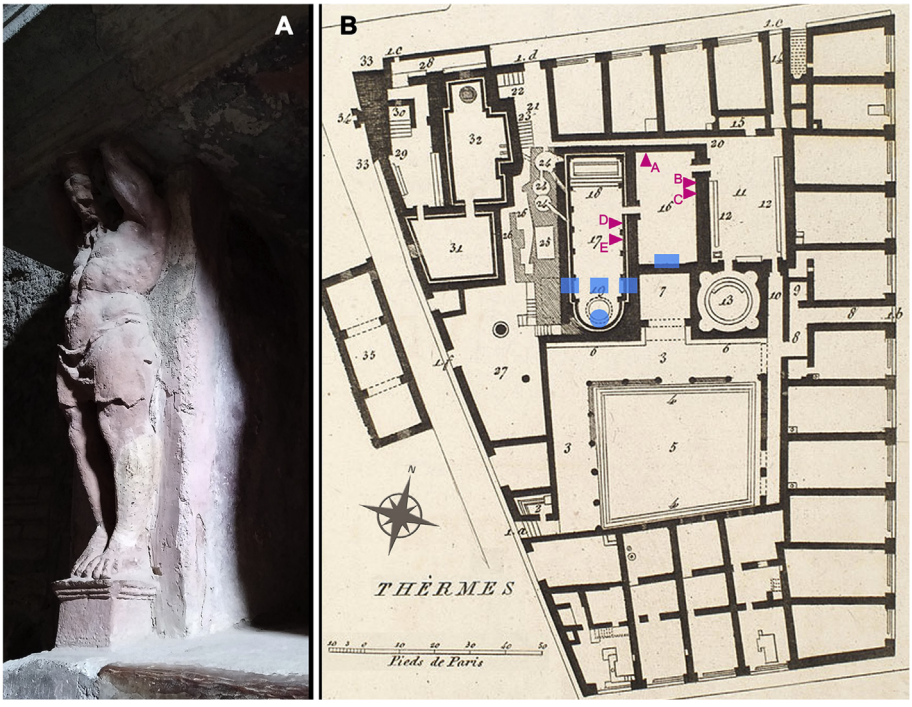

Fig. 1. Pink patinas and sampling points. A) Example of an Atlas displaying the pink patina (sample B) in *Tepidarium*. B) The layout of "*Terme del Foro*", skylights (blue shapes) and sampling points in *Tepidarium* (room 16, A to C) and *Calidarium* (room 17, D and E) are indicated (pink arrows) (de Jorio, 1836 copyright of image: Deutsches Archäologisches Institut in Rom). 1 *Pied de Paris* = 32.484 cm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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