

Case study

Bacterial community analysis on the Mediaeval stained glass window “Natività” in the Florence Cathedral

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Abstract

Microbial corrosion of glass causes problems on delicate antique glass samples. Until now, the effect of microbial activity on corrosion phenomena has not been well documented. Only a few studies have been published concerning the microflora growing on glass surfaces.

The present study deals with the characterization of cultivable aerobic bacteria isolated from the historical glass window “Natività” in the Florence Cathedral, designed by Paolo Uccello and realized by Angelo Lippi between 1443 and 1444. Microbial strains were sampled from four of the 25 panels of the “Natività” in the occasion of a recent conservation treatment, due to the presence of various kinds of crusts. One hundred microorganisms were isolated, about 50% bacteria and 50% fungi. Bacteria were submitted to morphological characterization and classified in the Gram group. For twenty strains, from different glass panels, the 16S rDNA gene was amplified and sequenced. Sequence analysis showed genus *Bacillus*, *Arthrobacter* and *Paenibacillus* as the most representative. In particular *Bacillus* and *Paenibacillus* are crusts associated. Phylogenetic relationship among isolates was determined. Chemical analysis of the glass and crusts completed the study.

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1. Introduction

Glass, like any other material, is damaged by aging. Drastic changes in the appearance, colour, and structure occur on stained glasses which have a large surface exposed to the atmosphere. Physicochemical mechanisms of deterioration are known [1] and microorganisms could accelerate physicochemical phenomena leading to decay processes. Recently, various papers have addressed the role of microorganisms in glass biodeterioration. Microorganisms can enhance the deterioration process by excretion of chemically aggressive substances and by physical attack on the glass [2–6].

Microorganisms, furthermore, are able to acquire the elements needed for growth from the glass itself [2].

The microbial flora is usually the result of successive colonisations by different microorganisms. A variety of microorganisms, including lichens (*Diploica*, *Pertusaris*, *Lepraria* sp.), fungi (*Aspergillus* sp., *Penicillium* sp.) and bacteria (*Flexibacter* sp., *Nitrosospira* sp., *Arthrobacter* sp., *Streptomyces* sp., *Micrococcus* sp., *Frankia* sp., *Geodermatophilus* sp.) were shown to grow on the glass surface [4,5,7–10]. Moreover, it has been demonstrated that bacterial and fungal communities on biodeteriorated glass surfaces are much more complex than previously believed [10,11].

The study of microbial communities on mediaeval glass is interesting to understand the relationship between the microorganisms and the glass surface. Studies about microbial communities are useful for monitoring microorganisms after completion of the conservation procedure [12], they are also

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useful in identifying biocides able to eliminate microorganisms.

In the last fifteen years molecular techniques based on rDNA sequences have been used to identify microorganisms on artistic glasses [10,11].

In this paper we report the characterization of bacteria isolated from the historic window “Natività” in the Florence Cathedral.

The window “Natività” belongs to a wide iconographic plan to glorify the Virgin Mary. The window, of extreme artistic value, was designed by Paolo Uccello and created between 1443 and 1444 by Angelo Lippi. It is located in the north-west position, in the tambour under Brunelleschi’s dome in the Florence Cathedral.

The window has recently been dismantled and restored. Microorganisms were isolated from glasses before restoration and characterized. Chemical analysis of glass and crusts have completed this case study.

2. Materials and methods

2.1. Sampling and growth condition

Contact plates (BIOSTER) filled with Nutrient Agar (OXOID) with 1% glucose were used for microbiological sampling. Four of the twenty-five panels of the windows were

sampled with ten plates: panels 6, 14 and 17 on the outside surface of the window and panel 7 on the inside surface of the window (Fig. 1). On each panel, different glass type and crusts were chosen for sampling. The plates were incubated at room temperature for three days. Bacterial colonies were re-isolated several times, to obtain pure cultures, on Nutrient Agar medium. Strains were named by a number, corresponding to the panel, followed by a letter, corresponding to the sampled area of the panel, and by a last number identifying the strain.

2.2. Phenotypical characterization

Colonies were examined under the stereomicroscope in order to characterize their shape. Cell morphology was observed in fresh samples with a phase contrast Nikon Alphaphot YS microscope at 400 and 1000 magnifications. Lysis test was performed with KOH 3% (water solution) to classify the isolates into the Gram group [13].

2.3. Test of copper resistance [14]

Single colonies were inoculated in 10 ml Nutrient Broth and incubated at 28–37 °C with shaking at 150 rpm. Cells were counted in a Petroff Counting Chamber (Hausser Scientific Company). An aliquot (10 µl) of a cell suspension

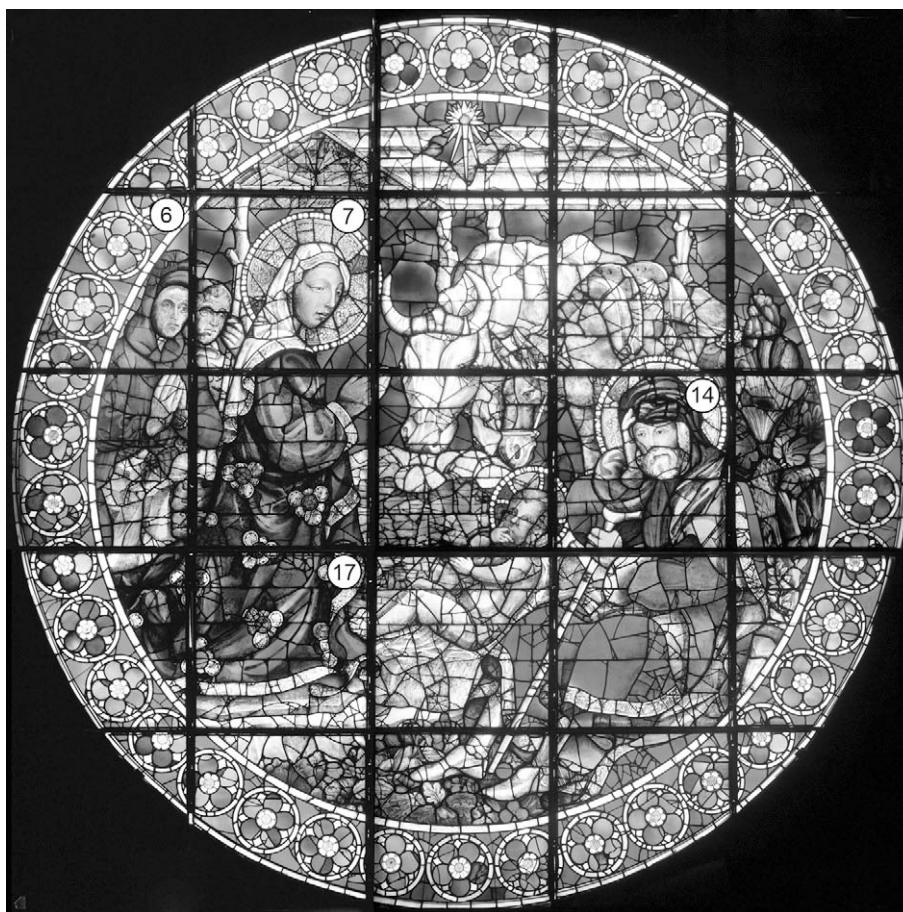


Fig. 1. Window of “Natività” in the Florence Cathedral. It was made between 1443 and 1444 by Angelo Lippi. It represents the birth of Jesus. The numbers represent the sampled panels.

containing 5×10^8 cells/ml was spotted on Nutrient Agar plates added with various concentration of CuSO_4 (0; 0.2; 0.4; 0.8; 1.6 and 3.2 mM). Plates were incubated at two different temperatures of 28 and 37 °C, depending on the growth conditions. Minimal inhibitory concentration (MIC) was expressed as the concentration that inhibited confluent growth of the culture after 24, 48 or 72 h (depending on the growth speed of strains). Each strain was tested at least twice.

2.4. Molecular techniques

To extract DNA, bacteria were grown on Nutrient Agar as confluent patina. DNA extraction was performed with FastDNA Kit (Q-BIOgene) according to the manufacturer's specifications.

The 16S rDNA amplification was performed using primers P0 and P6 which anneal to positions 8–27 and 1495–1515, respectively, of the *Escherichia coli* 16S rDNA gene [15]. Primers have a tail (underlined) of Universal M13 Forward primer preceding P6 (5'-GTAAAACGACGGCCAGTC-TACGGCTACCTTGTACGA-3') and of Universal M13 reverse primer preceding P0 (5'-CAGGAAACAGCTATGACC-GAGAGTTTGATCCTGGCTCAG-3').

A “touch-down PCR” program was used with annealing temperature decreasing in progressive cycles. PCR conditions consisted of an initial denaturing step of 5 min at 95 °C followed by 5 cycles of 95 °C for 30 s; 60 °C for 30 s, 72 °C for 2 min; 5 cycles of 95 °C for 30 s; 55 °C for 30 s, 72 °C for 2 min and 25 cycles of 95 °C for 30 s; 50 °C for 30 s and 72 °C for 2 min. The reaction was completed with a final extension at 72 °C for 10 min and then cooled and held at 8 °C. PCR was carried out with 1 unit of *EuroTaq* polymerase (Euroclone) on Primus Thermal Cycles machine (MWG). A small amount of all PCR products was analyzed by electrophoresis on 0.8% agarose gel in TBE buffer. All PCR products were purified with High Product Purification Kit (ROCHE).

Purified fragments were sequenced with Universal M13 Forward and Reverse primers by the analyzer ABIPrism310 (Applied BIOSYSTEM).

Nucleotide sequence accession numbers: the 16S rDNA sequences of twenty strains are available at the NCBI database under the accession numbers from AM900494 to AM900513.

2.5. Analysis of 16S rDNA

All sequences were analyzed with DS Gene software. They were compared at the prokaryotic small subunit rDNA Ribosomal Database Project II website (<http://rdp.cme.msu.edu/index.jsp>; [16]). “Classifier” option was used to assign them a taxonomical hierarchy. “Sequence Match” option was used to assign them the nearest neighbour sequences contained in RDP II. 16S rDNA sequences retrieved from databases were aligned with the MEGA version 4.0 software [17]. The resulting alignments were checked manually and corrected if necessary. The software MEGA 4.0 was used to construct phylogenetic trees. They were inferred using the neighbour-joining method [18]. Trees were unrooted. Sequence

divergences among strains were quantified by using the Kimura-2-parameter distance model [19]. For treatment of gaps “Complete Deletion” option was chosen. Bootstrap analysis (1000 replicates) was used to test the topology of the neighbour-joining method data.

2.6. Chemical analysis on green glass surfaces and on decay crusts

The green glass was analyzed by Scanning Electron Microscopy (SEM) coupled with Energy Dispersive X-rays (EDX) analysis. The two most common types of grey and red crusts on panels, besides of SEM/EDX, were characterized also by Fourier Transform InfraRed spectroscopy (FTIR). Two different regions of the green glasses were analyzed. The first named “external side”, referring to the surface that in the case of green glass was not affected by the crusts, and the second one named “thickness side”, that was the part of the glass where the lead bars (removed for the analysis) supported the glass.

For infrared measurements a Bio-Rad FTS 40 spectrometer was used; measurements were carried out in the frequency region from 4000 to 400 cm^{-1} with 4 cm^{-1} resolution and 32 scans. The samples (powder coming from external crusts present onto the glass) for infrared analysis were prepared according to the KBr pellet technique. Microscopic observation was done by SEM Cambridge stereoscan 360 fitted with an Oxford Instrument energy dispersive X-ray analyzer model INCAX-sight 7060 on the above mentioned glass regions after graphitisation.

3. Results

The window of “Natività” measures about 25 m^2 (Fig. 1), it is made with several stained pieces of glass and it is supported by lead bars. The pictorial decoration and the grisailles are inside the Cathedral. The window is cemented to the tambour structure with lead bars. The window was recently dismantled for restoration due to deterioration. The deterioration was linked to corrosion and (or) formation of neo-mineral phases, namely coloured crusts on the glass external surface.

On the outside of the window the glass panels showed a great and visible damage: rough surface, pitting, powder crusts and hard crusts (Fig. 2a); on the contrary, green glasses did not show visible crusts (Fig. 2b). On the inside of the window, stained glasses were in good condition and without macroscopic crusts.

3.1. Sampling and phenotypical characterization

Ten contact plates were used to sample four panels: panels 6, 14, 17 on the outside and panel 7 on the inside of the window. Panels 6 and 17 were sampled both on green glass and on stained (not green) glass. Plates were kept at room temperature for 3 days. One hundred microbial strains were isolated. They were divided into 47% bacteria, 50% moulds

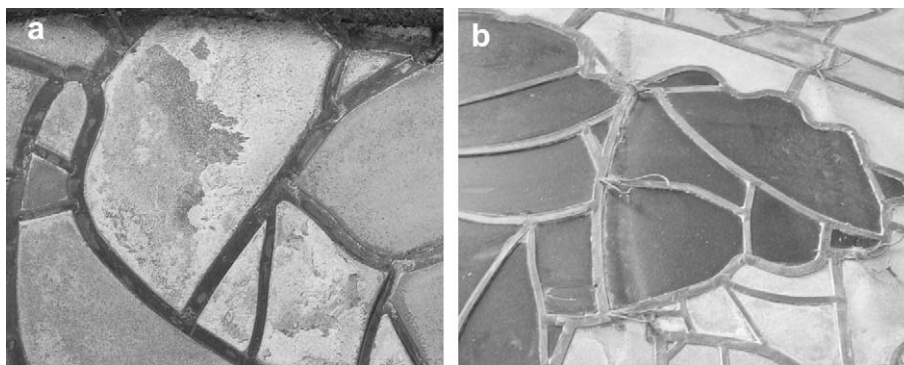


Fig. 2. Glass panels on the outside of the windows, (a) Stained glass showing grey crusts. (b) Green glass without macroscopic crusts.

and 3% yeasts. Bacteria only were further characterized in this work. Fig. 3 shows the results about the number of bacterial strains found on each panel. On green glass (6C, 17C) the number of isolated strains was lower than on other stained glass.

Observation at the phase contrast microscope showed that the most common cellular shape was rod (Fig. 4). The lysis test showed the preponderance of Gram positives (Fig. 4).

3.2. Molecular characterization

Genomic DNA was extracted from 20 strains. They were chosen with the following criteria: eight strains sampled from panel 6 (6A, not green glass) and all the three strains from green glass of panel 6 (6C); four strains from the inside of the Cathedral on panel 7; two strains from panel 14 and three strains from panel 17; for the last panel two strains are from green glass and the other one from not green glass. In this way, all strains sampled from panel 6 and at least two strains from each panel, were characterized. The 16S rDNA was amplified and sequenced. Sequences were compared with RDP II databases. Results of analysis, together with phenotypical data of strains are shown in Table 1. Genus assignment was possible for all the strains except 17C-7. It was possible to analyze only 200 bp of strain 17C-7 and to assign to it the order *Bacillales*.

The 16S rDNA sequences, with the exception of 17C-7, together with similar sequences from database were used to construct three phylogenetic trees to determine the

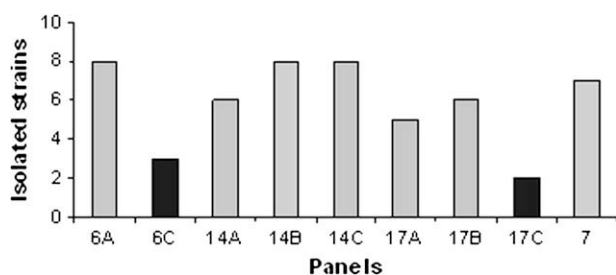


Fig. 3. Distribution of bacterial strains isolated from glass panels. Asics: panel areas sampled. The number corresponds to the panel, the following letter corresponds to different areas into the same panel. Ordinates: number of strains sampled. Strains isolated from green glass are in black columns (6C, 17C).

relationship among the strains. For each strain, the best matching sequences both from the “type” and “non type” strain (Table 1) were used for the phylogenetic tree (Fig. 5). When the “type” strain sequence had the best score, the best matching “non type” strain was not used. Almost all the recovered isolates belong to *Firmicutes* and *Actinobacteria* phyla and their relationships are shown in Fig. 5a, b.

The majority of isolates belongs to the Gram positive Low G + C group. Among these, there are all the strains (eight) sampled from non green glass of panel 6, almost all clustering with *Bacillus* related strains except for strain 6A-7 which clusters with *Paenibacillus* strains. Nevertheless, strains of panel 6 are scattered in 6 clusters showing a certain degree of divergence. 6A-9 and 6A-10 strains clustered with *Bacillus megaterium*. Strain 6A-8 with *Bacillus simplex*, strains 6A-6 and 17B-6 with *Bacillus thuringiensis*, strains 6A-1 and 6A-2 with *Bacillus mojavensis*, 6A-4 with *Bacillus pumilus*. 6A-7 and 6C-4 were phylogenetically related with *Paenibacillus pabuli*. The last strain of the low G + C group was 14A-7 and it clustered with *Paenibacillus polymyxa* (Fig. 5a).

Six isolates were related to representatives of the Gram positive High G + C group and belonged to different species of *Arthrobacter* and *Leucobacter* genera. 6C-1 and 7-7 were phylogenetically related to *Arthrobacter agilis*, strain 14C-5 to *Arthrobacter crystallopoietes* and 7-5 to *Leucobacter komagatae*. 6C-5 was phylogenetically related to *Leucobacter albus* and 7-3 to an *Arthrobacter* sp. strain (Fig. 5b).

The third tree shows the *Proteobacteria* phylum (Fig. 5c) including two Gram negative isolates. Strain 17C-6 was

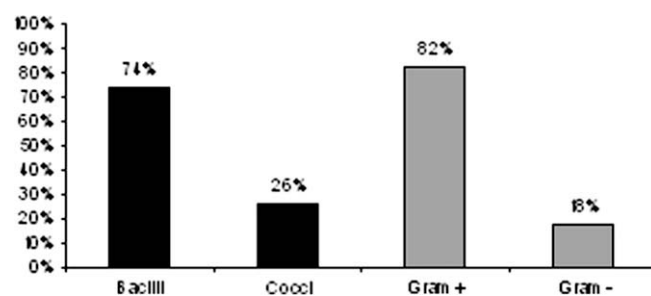


Fig. 4. Percentage of cocci, bacilli, Gram positives and Gram negatives among isolated bacteria.

Table 1
Phenotypical and molecular results of 20 isolated bacterial strains.

Strain	Cell morphology	Gram group ^a	16S rDNA sequence (bp)	Genus assignment ^b	Best match ^c
6A-1	Rods	+	1283	<i>Bacillus</i>	(1.000) 1435 <i>Bacillus mojavensis</i> IFO15718; AB021191 (0.916) 1463 <i>Bacillus licheniformis</i> (T) DSM 13; X68416
6A-2	Rods	+	1349	<i>Bacillus</i>	(0.982) 1435 <i>Bacillus mojavensis</i> IFO15718; AB021191 (0.953) 1440 <i>Bacillus vallismortis</i> (T); DSM11031; AB021198
6A-4	Rods	+	1427	<i>Bacillus</i>	(0.982) 1261 <i>Bacillus pumilus</i> WN697; AY260859 (0.874) 1440 <i>Bacillus vallismortis</i> (T); DSM11031; AB021198
6A-6	Streptobacilli	+	1326	<i>Bacillus</i>	(0.983) 1406 <i>Bacillus thuringiensis</i> (T); ATCC10792; AF290545
6A-7	Rods	–	1393	<i>Paenibacillus</i>	(0.981) 1406 <i>Paenibacillus pabuli</i> HSCC 473 (NRRL BD-537)AB045104 (0.969) 1402 <i>Paenibacillus amylolyticus</i> (T); NRRL NRS-290T; D85396
6A-8	Rods	+	1293	<i>Bacillus</i>	(0.965) 1418 <i>Bacillus simplex</i> LMG 21002; AJ628745 (0.853) 1034 <i>Bacillus simplex</i> (T); DSM 1321; X60638
6A-9	Streptobacilli	+	1454	<i>Bacillus</i>	(0.999) 1423 <i>Bacillus megaterium</i> MO31; AY553118 (0.939) 1030 <i>Bacillus megaterium</i> (T); DSM 32; X60629
6A-10	Streptobacilli	+	1396	<i>Bacillus</i>	(0.998) 1425 <i>Bacillus megaterium</i> GSP10; AY505510 (0.940) 1352 <i>Bacillus simplex</i> (T) DSM 1321; D78478
6C-1 (green glass)	Diplococci	+	1444	<i>Arthrobacter</i>	(0.972) 1394 <i>Arthrobacter agilis</i> (T) DSM 20550; X80748
6C-4 (green glass)	Rods	+	1359	<i>Paenibacillus</i>	(0.961) 1384 <i>Paenibacillus amylolyticus</i> JCM 9906 AB073190 (0.954) 1402 <i>Paenibacillus amylolyticus</i> (T) NRRL NRS-290T; D85396
6C-5 (green glass)	Cocci	+	1197	<i>Leucobacter</i>	(0.962) 1415 <i>Leucobacter albus</i> IAM 14851; AB012594 (0.920) 1400 <i>Leucobacter komagatae</i> (T) JCM 9414; D45063
7-3 (inside)	Cocci	+	1389	<i>Arthrobacter</i>	(0.989) 1319 <i>Arthrobacter</i> sp. (T) gifu12498; D88211
7-4 (inside)	Rods	–	1296	<i>Brevundimonas</i>	(0.937) 1347 <i>Brevundimonas subvibrioides</i> (T) LMG 14903T; AJ227784 (0.914) 1343 <i>Brevundimonas variabilis</i> (T) ATCC 15255 (T); AJ227783
7-5 (inside)	Cocci	+	1226	<i>Leucobacter</i>	(0.983) 1430 <i>Leucobacter komagatae</i> IFO15245T; AJ746337 (0.953) 1400 <i>Leucobacter komagatae</i> (T); JCM 9414; D45063
7-7 (inside)	Cocci	+	1370	<i>Arthrobacter</i>	(0.948) 1394 <i>Arthrobacter agilis</i> (T) DSM 20550; X80748
14A-7	Rods	+	1448	<i>Paenibacillus</i>	(0.971) 1358 <i>Paenibacillus polymyxa</i> GBR-27; AY359615 (0.954) 1411 <i>Paenibacillus kribbensis</i> (T) AM49; AF391123
14C-5	Cocci	+	1350	<i>Arthrobacter</i>	(0.984) 1393 <i>Arthrobacter crystallopoietes</i> (T) DSM 20117; X80738
17B-6	Streptobacilli	+	1390	<i>Bacillus</i>	(0.981) 1406 <i>Bacillus thuringiensis</i> (T) ATCC10792; AF290545
17C-6 (green glass)	Streptobacilli	–	1420	<i>Stenotrophomonas</i>	(0.959) 1384 <i>Stenotrophomonas maltophilia</i> (T) ATCC 13637T; AB008509
17C-7 (green glass)	Streptobacilli	+	200	<i>Bacillales</i> ^d	(0.550) 1483 <i>Bacillus firmus</i> (T) IAM12464; D16268 (0.594) 1474 uncultured soil bacterium 432-1; AF423263

^a Gram group was assigned by lysis test.

^b Genus assignment by the Classifier option.

^c In parenthesis, the similarity score assignment by the Sequence Match option: the number of (unique) oligomers shared between query sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences. The number after similarity score is the full-length sequence.

^d Order assignment.

phylogenetically related to *Stenotrophomonas maltophilia* belonging to *Gammaproteobacteria*, while strain 7-4 was phylogenetically related to *Brevundimonas subvibrioides*, belonging to *Alfaproteobacteria*.

3.3. Chemical analysis

The chemical composition of the green glass is shown in Table 2. The concentration of the main network-forming SiO_2 is about 60% on both regions of the glass (external and thickness sides; see 2.6). The network-modifying alkaline oxide Na_2O in the two sites ranges between 17 and 20%, whereas the concentration of K_2O is about 4% for both samples. Network-stabilizer alkaline-earth oxide CaO was about 6.6% in both. The percentage of CuO ranges between 1.21 and 1.87%. The lead was present only on the “thickness side” sample in the 0.71% (as PbO_2 wt%) and not on the “external side”. The discussion about the differences between

these two samples will follow in the fourth section. Concerning the analysis of the crusts, two different stained crusts, grey and red, were present on the not green glass outside of the window (Fig. 2a). The results of crust analysis is shown in Table 2. Sulphur, potassium and calcium are the most abundant elements. FTIR spectra of the two crusts shows the presence of calcium and potassium sulphate (Fig. 6). In particular, FTIR spectra individuated selenite (gypsum) and syngenite.

3.4. Copper resistance

No macroscopic crusts were found on green glasses. Moreover, the lowest number of strains was recovered from the green glass (Fig. 3). This phenomenon could be due to the presence of a microbial inhibitor. Chemical analysis shows the presence of copper (Table 2), that was used in the past as network modifier able to stain the glass as green during the

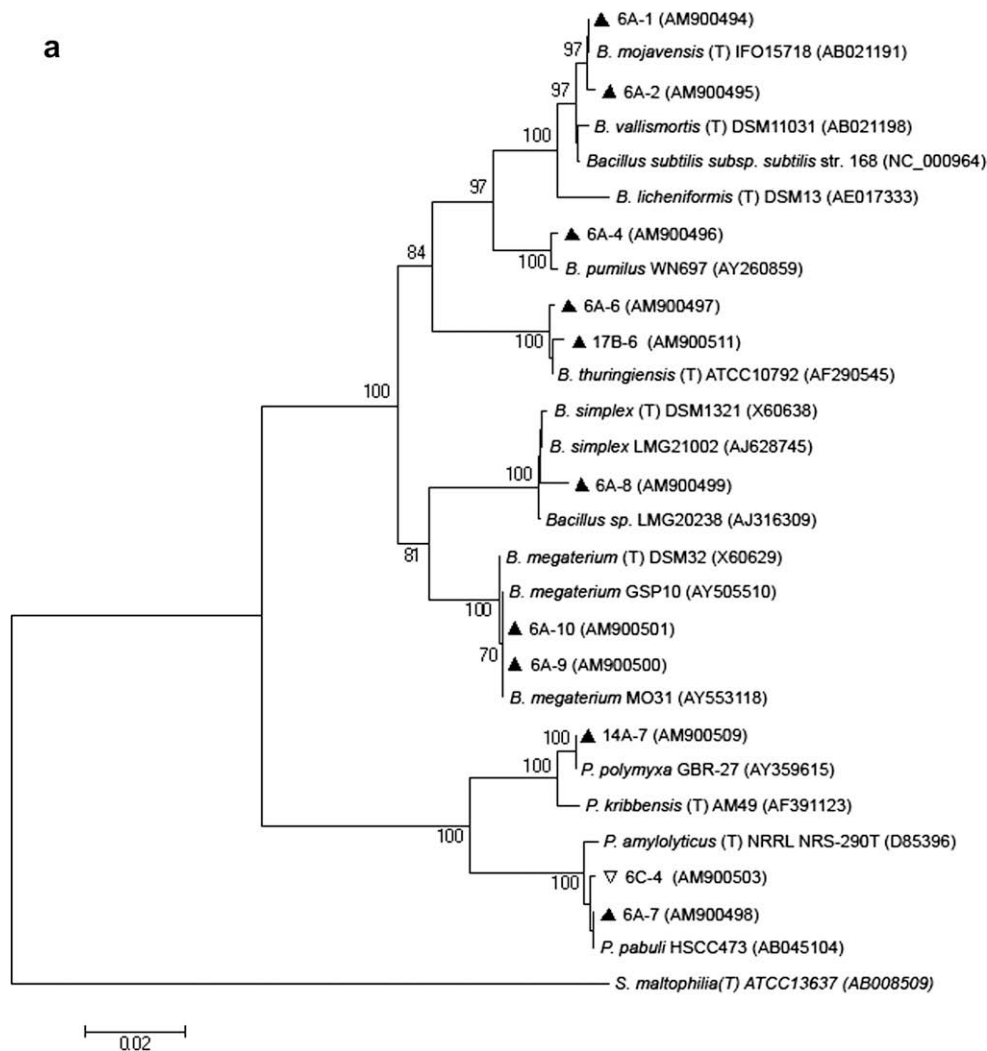


Fig. 5. Phylogenetic relationship of isolated strains. Numbers indicate bootstrap percent confidence. The scale bar indicates substitutions per nucleotide. The accession numbers for the 16S rRNA sequences are reported after the strain name in parenthesis. Black triangles represent strains sampled on glass with macroscopic crusts; white triangles represent strains sampled on glass without macroscopic crusts (inside the Cathedral and on green glass). *S. maltophilia* (T) ATCC 13637T in a) and b) and *B. subtilis* subsp. *subtilis* strain 168 (T) in c) were used as out-group. a) Tree of strains belonging to Class Bacilli (Phylum Firmicutes). b) Tree of strains belonging to Class Actinobacteria (Phylum Actinobacteria). c) Tree of strains belonging to Class Proteobacteria Phylum.

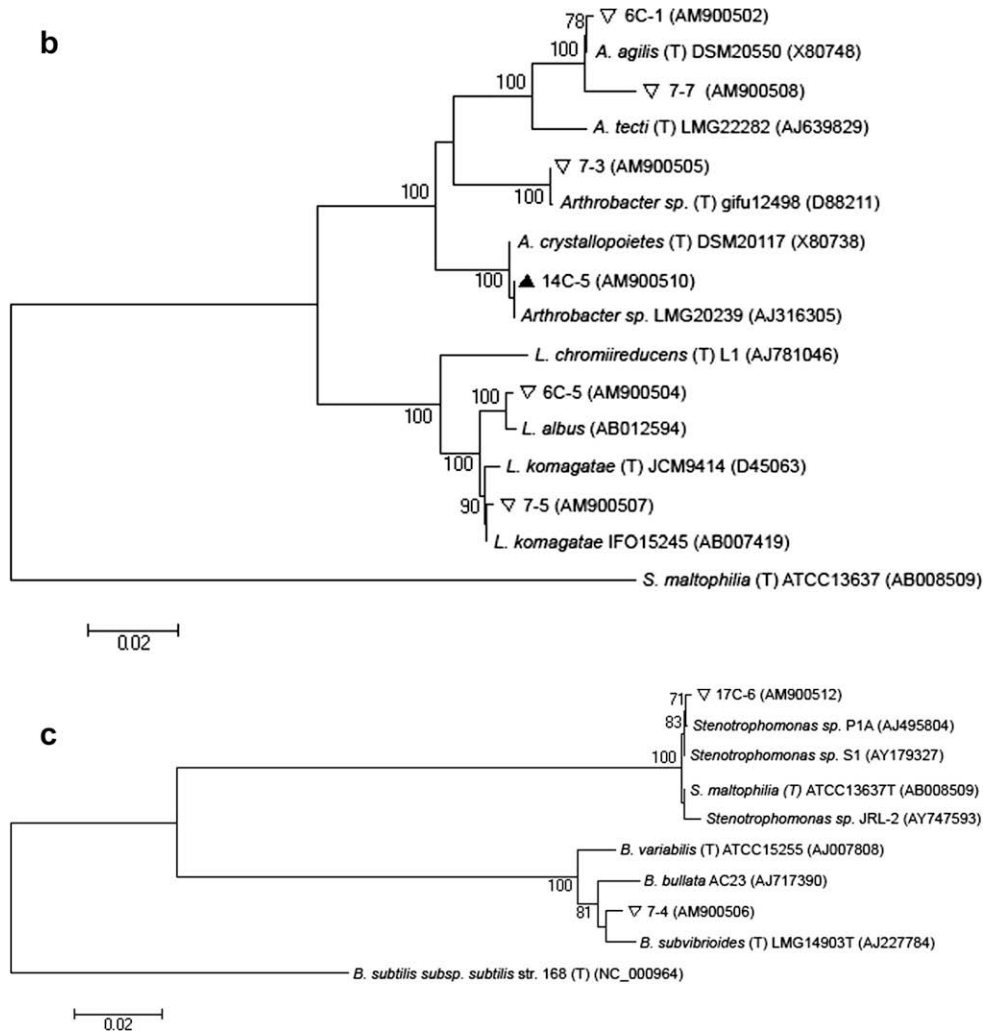


Fig. 5. (continued).

fusion processes [20]. It is known that copper (1–10 μM) in its free ionic form (Cu^{2+}) is toxic to microbial cells [21]. Since copper was present in green glass we tested if these strains were more resistant to copper than other isolated from crusts by determination of the MIC. *Bacillus subtilis* 168 and some strains sampled from panel 6 were also tested as control.

For each strain 10 μl suspension containing 5×10^6 cells were spotted on Nutrient Agar with different concentrations of CuSO_4 . Results are shown in Table 3. No difference in MIC values was found between strains sampled from green glasses and the others.

We remind even though lead is toxic for microorganisms, our investigation showed that lead was not detected on “external side”. Traces of lead were present only on the “thickness side” from the regions where lead bars were in contact with the glass.

4. Discussion

In Spring 2004 conservators took out the window “Natività” from the Florence Cathedral in order to clean the stained

glass, 50 years after the last conservation intervention. Macroscopic crusts covered a large part of the glasses with the exception of the inside face and the green glass pieces.

4.1. Chemical analysis

Chemical analysis was made to determine the chemical composition of crusts and green glass.

The chemical analysis of green glass was done both on the “thickness side” and on the “external side”. On both sides Si content (expressed as SiO_2) was greater than 60% (Table 2) and Na (expressed as oxide) ranged between 17 and 20%, showing a typical alkaline silicate-sodium glass of the Mediaeval-Renaissance period. In fact, in literature, two main compositional groups of antique glasses can be distinguished: Na-rich and K-rich glasses. Na-rich glasses typically show greater silica content (55–65%) than the K-rich glasses. Garcia-Vallès et al. [22] found similar values to our glass about Si, Na, Ca and Cu percentage in a 13th–15th centuries dark-green glass from Pedralbes Monastery (Barcelona).

Table 2

Chemical composition of the glass and crust samples as determined by EDX and expressed as the corresponding oxides.

Oxides	Wt%	Wt%	Wt%	Wt%
	Green glass “external side”	Green glass “thickness side”	Grey crust	Dark-red crust
Na ₂ O	17.45	20.35	0.19	—
MgO	3.91	3.31	0.48	0.83
Al ₂ O ₃	1.30	1.19	—	—
SiO ₂	60.42	60.0	7.92	15.30
SO ₃	1.39	—	35.88	38.20
ClO ₂	2.16	—	—	—
K ₂ O	3.68	4.25	41.83	25.79
CaO	6.51	6.67	13.71	15.37
Fe ₂ O ₃	1.27	2.28	—	1.51
CuO	1.87	1.21	—	—
PbO ₂	—	0.71	—	—

FTIR crusts analysis shows the presence of gypsum (selenite, CaSO₄·2H₂O) and syngenite (K₂Ca(SO₄)₂·H₂O). The peak at 1383 cm⁻¹ attributed to the anion NO₃⁻ establishes the presence of low amount of nitrates: it is impossible, with such low quantity, to determine the type of salt. EDX analysis, in particular, reveals the presence of iron on the red crust (Table 2), probably associated to iron oxides used for colouring the glass. Concerning the chemical composition of both green glasses and crusts coming from not green glasses we can draw the following conclusion: (i) the green glass was not stabilized by lead since this element was found only in the regions (thickness side) where lead bars had been present; (ii) the green glasses were much more stable than not green ones with less corrosion phenomena; (iii) the chemical nature of the crusts onto not green glass (gypsum and syngenite) infers that these glasses were subjected to heavy corrosion phenomena, alkaline conditions and crust formation.

The only light corrosion phenomena observed on the green glasses are deducible from SEM/EDX (see Table 2) where the exposed surface (“external side”) presents S and Cl elements even though at low concentration. It was impossible

Table 3

MIC of CuSO₄. MIC was determined respectively after 24 h (3) at 28 °C, 48 h (2) at 28 °C and 24 h (1) at 37 °C, depending on growth speed of each strain.

Strain	MIC CuSO ₄ (mM)
<i>B. subtilis</i> strain 168	1.6 ¹
6A-1	0.8 ¹
6A-2	0.8 ¹
6A-4	1.6 ¹
6A-10	0.8 ³
6C-1	1.6 ²
6C-4	0.4 ¹
6C-5	0.8 ¹
17C-6	1.6 ¹
17C-7	1.6 ¹

to make FTIR spectra due to the absence of a true crust to be sampled.

4.2. Bacterial community

Before restoration, touch plates were used to sample and isolate cultivable aerobic bacteria from glass and crusts. One hundred microorganisms were isolated, 47% of all isolated strains were bacteria.

Bacteria present on the historical window were further characterized by classical and molecular microbiological techniques. Classical techniques as microscope and physiological investigations showed that bacilli and Gram positive bacteria were dominant. A dominance of Gram positives was also found on other cultural heritage substrates as damaged frescoes [23]. Molecular results, based on 16S rDNA analysis of 20 strains, showed that the related features of the assigned genera are in agreement with the phenotypical data (Gram classification and cell morphology, Table 1). In the case of strain 6A-7, it should belong to *Paenibacillus* genus and to the Gram negative group. On the other hand, in literature, *Paenibacillus* genus shows heterogeneity for Gram staining; some species are Gram positive, other Gram negative and other species have a variable reaction depending upon growth stages [24,25].

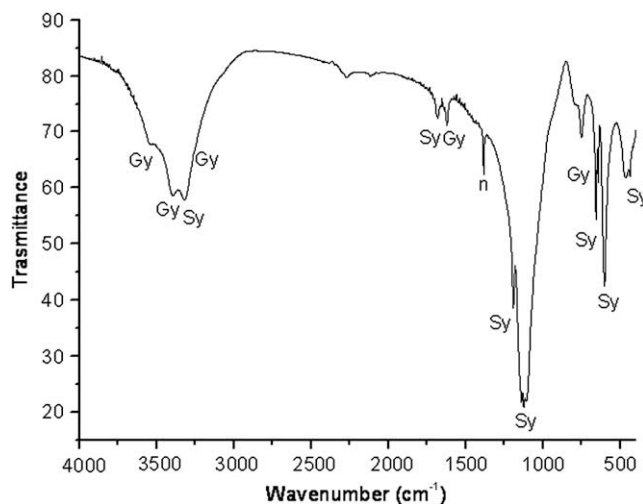


Fig. 6. FTIR spectrum of the grey crust: the typical absorption of gypsum (gy), syngenite (sy), and nitrates (n) are labeled.

Almost all strains found on the external glasses, except 14C-5, are clustered into *Firmicutes* phylum. *Firmicutes* were not isolated on the inside of the window; this suggests a different microbial presence in the confined atmosphere inside the Cathedral. All the *Firmicutes* strains are different (Fig. 5a, b), with the possible exception of strains 6A-9 and 6A-10. Their rDNA sequences are quite identical with a difference of only 2 bases; therefore they could be the same strain. *Firmicutes* were distributed mainly on panel 6 and, with the exception of strain 6C-4, on the stained glasses with presence of macroscopic crusts (Fig. 5a, black triangles). Since *Bacillus* and *Paenibacillus* genera are able to generate spores, this could suggest the ability to survive for a long time on the crusts. On the clean glasses (green glass and inside glasses) we found only one strain (6C-4) or possibly two (17C-7) of *Firmicutes*, and 5 not *Firmicutes* strains (6C1, 6C-5, 7-3, 7-5, 7-7) which are not sporulating genera belonging to *Actinobacteria* [26,27]. Not sporulating genera *Actinobacteria*, have already been found on antique glass: they were isolated on glass samples from the Cologne Cathedral [28] and *Arthrobacter* was found by DGGE-analysis of 16S rDNA extracted from glass samples in a German Protestant chapel in Stockkämper [10]. Among strains we isolated, 7-7 has a rDNA sequence similar to that of *Arthrobacter tecti* (type strain LMG 22282) isolated from deteriorated mural paintings [29]. Regarding *Proteobacteria*, sporulation is unclear for *Stenotrophomonas* and *Brevundimonas*; especially *Brevundimonas* is often specialized for oligotrophic [30] or alkaline environments (as *Brevundimonas bullata* AC23 which has the rDNA sequence similar to that of 7-4; Fig. 5c). To our knowledge, *Brevundimonas* and *Stenotrophomonas* have never been found on antique stained glasses.

Even if strains isolated from green glass were few, nevertheless they showed the greater variability. The four strains well characterized (6C-1, 6C-4, 6C-5, 17C-6) belong to four different genera (for 17C-7 it was not possible to assign a genus) and are distributed into all the three phyla: *Firmicutes*, *Actinobacteria* and *Proteobacteria*. The few number of strains does not allow to infer conclusions on this aspect.

4.3. Green glass vs. crusts

The green glass was almost clean, no macroscopic crusts were present on its surface and moreover we found a low amount of microorganisms. These observations could be in agreement with the presence of a microbial inhibitor. Chemical analysis showed that green glass contains copper (1.21–1.87%). Copper in its free ionic form (Cu^{2+}) is toxic to microbial cells [21] and in some cases Cu-rich portion of the glass acts as a barrier to bioactivity [9,22]. Similar results are described by Milanesi et al. (2006) [31] who found that high concentration of copper carbonate hydrate in mediaeval wall frescos can reduce the biodiversity of microorganisms in the pictorial surface. Copper is an element that cannot act as a glass network former, and in K-rich glass it is released [32]. We wondered if the microorganisms found on the green glass

were particularly resistant to copper by testing them by M.I.C. assay. A soluble copper salt (CuSO_4) was used in order to have free Cu^{2+} into the media. No substantial difference was found among strains found on green glass and other strains found on stained glasses (Table 3). This result excludes a particular copper resistance of the green glass isolates, and suggests other inhibition mechanisms. Microbial growth inhibition might be due to lead toxicity: indeed, this metal was not present on the “external side” but the lead bars could affect microorganisms viability.

Anyhow, the green glass, due to its intrinsic features of durability (Na-rich glass), does not allow the crusts development and, consequently, the presence of microorganisms in large quantities.

In the case for the K-Ca- SO_4 crusts, where we found a larger number of microorganisms, the situation is different. Here bacteria could find a more protected and favourable environment compared to the green glass surface. Crusts can trap airborne particulate, organic matter and environmental microorganisms, in particular spore forming bacteria. Moreover, crusts could retain moisture, protecting microorganisms from the high temperatures that glasses reach during sun irradiation.

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