ELSEVIER

International Biodeterioration & Biodegradation 57 (2006) 168–173

INTERNATIONAL BIODETERIORATION & BIODEGRADATION

www.elsevier.com/locate/ibiod

Biodeterioration of a fresco by biofilm forming bacteria

Claudio Milanesi[a,*], Franco Baldi[b], Sara Borin[c], Rita Vignani[a], Fabrizio Ciampolini[c], Claudia Faleri[a], Mauro Cresti[a]

[a]*Department of Environmental Science 'G. Sarfatti', University of Siena, P.A. Mattioli 4, 53100 Siena, Italy*
[b]*Department of Environmental Science, Cà Foscari University, Calle Larga S. Marta, 30121 Venezia, Italy*
[c]*Department of Food Science and Technology, University of Milan, Celoria 2, 20133 Milan Italy*

Abstract

Three areas of the 18th century fresco by Sebastiano Conca in the apse of the church (Santissima Annunziata, Siena, Italy) showed signs of deterioration. Twelve small fragments with a total weight of about 15 mg were detached from each of three damaged areas of the fresco and analysed. Element composition of specimens was determined by scanning electron microscopy (SEM) with energy dispersive X-ray analysis (EDX). Some fragments were incubated in mineral medium without organic carbon. Samples from the lower part of the fresco (area A) produced colonies of heterotrophic aerobic bacteria on the carbon available in this medium. Fragments from two other two areas (areas B and C) did not produce any microorganisms. Two strains were isolated from area A and identified as *Kocuria erythromyxa* strain CV1 and *Sphingomonas echinoides* strain CV2 by sequencing gene 16S rDNA. When fresco fragments were soaked in mineral medium without carbon source, *Sphigomonas echinoides* grew rapidly. This strain produced a visible biofilm on the fresco fragments. The mucilage consisted mainly of extracellular polymeric substances (EPS) detected in situ by confocal scanning laser microscopy (CSLM) with Con-A fluorescent lectin and confirmed by transmission electron microscopy (TEM) observations. Ultrastructural analysis of sections of the microorganisms by TEM showed that only strain CV2 adhered to the substrate and caused biological deterioration of the fresco.

Keywords: Biodeterioration; Endogenous carbon; Extracellular polymeric substances; *Kocuria erythromyxa*; *Sphingomonas echinoides*

1. Introduction

Mortar and plaster are readily colonised by virtue of their porous nature and mineral composition. Interaction with the atmosphere may cause deposition of particulate containing microorganisms. These are found in greater quantities near windows and doors, indicating the influence of air circulation (Gorbushina and Petersen, 2000; Saarela et al., 2004). An increase in the concentration of quiescent heterotrophic aerobic bacteria does not necessarily cause biodeterioration of frescoes (Eggins and Oxley, 2001). Deterioration processes may be triggered by exogenous factors (Zanotti and Mandrioli, 1979). For example, humidity, poor ventilation and light (including artificial light) facilitate formation of biofilms composed of viscous EPS, which are hydrogels consisting of about 98% water, that adhere to the surface (Morton and Surman, 1994). Hydration and formation of biofilms that collect particles and atmospheric contaminants accelerate chemical corrosion (Warscheid and Braams, 2000; Beech, 2004) with oxidation, reduction and transformation of metal ions and pigment changes (Urzì and Realini, 1998). Uncontrolled development of this phenomenon often coexists with saline efflorescences, also promoted by high humidity (Gorbushina et al., 2004). The presence of salts may influence the properties of EPS by decreasing their viscosity and causing surface irregularities which are preferential sites for colonisation by various communities of microorganisms (Roldán et al., 2003) and make frescoes bioreceptive (Giullitte, 1995), increasing microbial concentration and diversification and

*Corresponding author.
E-mail address: milanesi@unisi.it (C. Milanesi).

0964-8305/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibiod.2006.02.005

causing detachment of the paint layer (Ciferri, 1999). The presence of sulphur also increases the proliferation of microorganisms (Overmann and Gemerden, 2000).

Culture and molecular techniques now make it possible to isolate and identify microorganisms that colonise frescoes but they do not confirm the biological origin of the damage (Ausubel et al., 1994; Heyrman et al., 2004). Most are from the original fresco constituents, such as inorganic pigments diluted with calcium carbonate, enriched with airborne nutrients during centuries of exposure (Ciferri, 1999). The heterothrophic microbial biocenosis of historical frescoes may be affected by the mixture of different endogenous nutrients but the nature of specific compounds on the fresco is unknown and often impossible to determine because by law (for example, Italian law Normal DL 3/80, 1980), only tiny samples can be taken from national heritage paintings. It is also difficult to reproduce the original composition of nutrients in vitro, unless the original carbon source from the fresco is used.

The aim of this research was to isolate culturable microorganisms on endogenous mixture of carbon sources present on the fresco fragments, in order to study them for insights into the causes of fresco biodeterioration. We were interested to know which bacteria could grow on the endogenous nutrients under uncontrolled humidity and whether their growth could damage the fresco. Our hypothesis was that some microorganisms which damage fresco grow on the carbon and other nutrients of the surface.

2. Materials and methods

2.1. Sampling of fresco

Sampling was performed according to Italian legislative procedure (DL Normal 3/1980). Specimens were obtained in three damage areas (A, B, C) of the 18th century fresco by Sebastiano Conca in the apse of the church Santissima Annuziata in Siena (Italy) (Fig. 1). Twelve irregular fragments from each deteriorated area were obtained: A, 1.8 m above floor level; B and C, 6 m above floor level. Total sample weight from each site was about 15.3 ± 3.4 mg. In collaboration with restorers, fragments were detached from the fresco using sterile tweezers before restoration was begun. All samples were placed in sterile plastic tubes, numbered and analysed within 24 h.

2.2. Element analysis in fresco samples by SEM-EDX

Two of each set of 12 fragments from areas A–C were prepared for analysis of element composition by SEM-EDX. Two incubated fragments from each site were also analysed. Incubation procedure is described in 2.3. Specimens were glued to standard vacuum-clean stubs and coated with graphite (Edwards, carbon scancoat, S150A). They were observed by SEM (Philips XL20). The instrument was equipped with an EDX DX4 probe which we used at 20 kV acceleration voltage to determine the elements in the fresco surface. Concentrations had an error of about 1%. Mean concentrations and standard deviations of each element were calculated from five random determinations at different spots on the samples. The X-ray beam was 4 μm wide and penetrated to a depth of 2 μm.

2.3. Isolation of microorganisms

In order to enrich the bacterial population thriving on endogenous carbon as carbon and energy source, each damaged area (measuring 20×20 cm) was wiped with a sterile cotton swab. The tip of the swab was

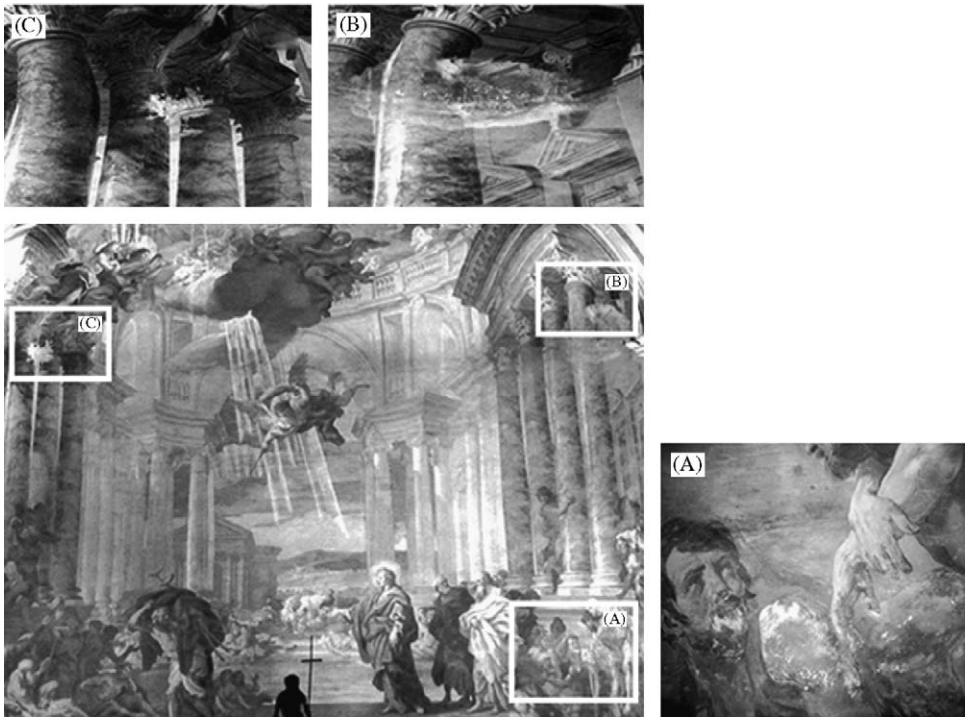

Fig. 1. Eighteenth century fresco in the apse of the medieval chapel of the Santissima Annunziata, Siena, showing damaged areas A–C where fragments were sampled for analysis.

cut off and incubated at 28 °C in test tubes with 10 of the fragments from the corresponding damage area in 10 ml mineral medium containing per litre: 1 g $MgSO_4.7H_2O$, 0.7 g KCl, 2 g KH_2PO_4, 3 g Na_2HPO_4, 1 g NH_4NO_3, at pH 6.7 (Baldi et al., 2003; Milanesi et al., 2006).

Only the culture from area A was visibly positive for bacterial growth after 18 h. Those from areas B and C were negative for bacterial growth even after 72 h of incubation. This was confirmed by light microscopy (LM) observation. Two of the 10 incubated fragments from site A were transferred with sterile tweezers to complete nutrient agar broth containing per litre of distilled water: 5 g peptone (Merck, Darmstadt, Germany), 3 g meat extract (Chemex, Munich, Germany) and 15% agar (Difco) at pH 6.7. Only two strains, identified as CV1 and CV2, were isolated from the medium. They were repeatedly smeared until final isolation and then stored in test tubes at −80 °C.

2.4. Identification of bacteria

The strains isolated from the complete nutrient agar were identified by sequencing the 16S rRNA gene. Briefly, fresh cells grown on complete nutrient agar were harvested and suspended in buffer consisting of Tris 10 mM and EDTA 1 mM, pH 8. Total DNA was extracted from each strain by the method of Ausubel et al. (1994). The 16S rRNA was amplified with the universal bacterial primers 27f (5′-agagtttgatcctggctcag-3′) and 1492r (5′-ctacggctaccttgttacga-3′). The reaction mixture contained per 100 µl: 0.3 µM of each primer, 2 U Taq Polymerase (Amersham Biotech, Milan, Italy) with the 1X buffer supplied, 0.12 mM dNTPs (Amersham Biotech, Milan, Italy) and 0.2 µl extracted DNA (approximately 100 ng). Polymerase chain reaction (PCR) was performed in a Bio-Rad I-Cycler thermal cycler (Bio-Rad, Milan, Italy). The 1450 bp PCR products obtained were purified by the QuiaQuick PCR purification kit (Quiagen, Milan, Italy) following the manufacturer's instructions and directly sequenced in separate reactions with the primers 27f, 1492r and 1100r (3′-agggttgcgctcgttg-5′) (Primm, Milan, Italy). The consensus 1300 bp was aligned in GenBank database, retrieving the nearest relatives for each sequence. A 100-times bootstrapped neighbour-joining tree was constructed on the sequences obtained with the Treecon program using the Kimura 2 parameter evolutionary model. The sequences obtained in the present study are available in GenBank under accessions nos. DQ176452, and DQ176453.

2.5. Biofilm observation by CSLM

Four of the 10 fragments from area A incubated in mineral medium were prepared to study biofilm formation on the fresco. Image analysis was performed by CSLM (BioRad model Microradiance MRAG1). The fluorescent molecular probe was the lectin concanavalin A (ConA) from *Canavalia ensiformis* (Jack bean) labelled with fluorescein isothiocyanate (FITC) (Sigma). ConA has an affinity for glucose and mannose residues. The distribution of the fluorescent probes in the fresco fragments was observed by LM and CSLM after 2 h incubation with the fluorochrome. To exclude autoflorescence of specimens, a control fresco material was previously incubated for 30 min with 1 M glucose solution, which inhibits lectin binding to substrate (Baldi et al., 2003). The specimen was washed twice and incubated for 2 h with ConA–FITC to demonstrate the specificity of ConA for mannose and glucose residues in the samples.

2.6. TEM observations

Two more of the 10 fragments incubated in mineral medium were prepared for TEM analysis to determine ultrastructural interactions between bacteria and the inorganic substrate of the fresco. Specimens were fixed in 3% glutaraldehyde for 30 min and 1% osmium tetroxide for the same time and dehydrated in 40%, 60%, 80%, 100% ethanol at room temperature. The samples were gradually infiltrated in Spurr's low viscosity embedding resin mixture (Spurr, 1969) and polymerized at 70 °C for 7 h. After polymerization, the blocks were sectioned with an LKB III ultramicrotome equipped with a diamond knife. Thin sections were collected on copper grids, stained for 3 min in 2% uranyl acetate and 2% lead citrate and observed in a Philips Morgagni 268D TEM.

3. Results and discussion

3.1. Elemental analysis and SEM observation of fresco fragments

The three damage areas of the fresco had rendering in two layers, typical of the fresco technique: a coarse underlying base and a surface film containing pigments. The artist dilutes pigments with calcium carbonate and washes them over the base before it is completely dry. The tempera does not penetrate very deeply and the fresco surface remains rough.

Study of the alterations caused by microbe colonisation by SEM/EDX indicated the elements present in the

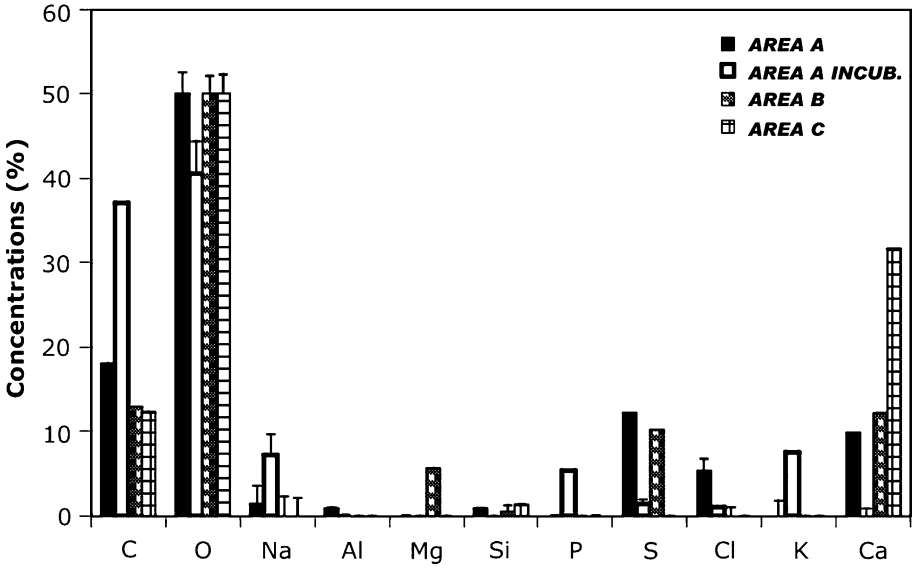

Fig. 2. SEM/EDX analysis of fresco samples without incubation from areas A–C and samples from area A incubated in mineral medium.

fragments (Fig. 2). Total carbon and sulphur concentrations showed different distribution in the three samples. Other minor elements were present. Total oxygen and calcium, typical of the calcium carbonate plaster used as base for the fresco, had similar percentages. Samples B and C contained 5.09% and 5.76%, respectively, of total carbon, and sample A contained 18%. Sample A also contained high concentrations of sulphur (12%) and chlorine (5%) with sodium (1.4%), aluminium (0.91%) and silicon (0.9%). Sample B had a high sulphur content (10.2%) and also contained magnesium (5.6%) with silicon (0.5%), but no aluminium. Sample C contained significantly lower total sulphur concentrations (1.3%) with traces of silicon (0.45%).

SEM observations of the fragments from area A (Fig. 3A), confirmed high porosity with clear signs of microbe adhesion (M) and penetration of the substrate. Fresco of sample from area B, rich in total sulphur, was mainly damaged by prolonged exposure to humidity from underlying layers where ashlars and bricks had been assembled with cement mortar. At high humidity, soluble calcium carbonate is transformed into calcium sulphate, namely gypsum ($CaSO_4 \cdot 2H_2O$) (Strzelczyk, 1981; Atlas et al., 1988) which discolors the fresco and causes visible salt efflorescence. SEM observation confirmed that the fragments were rich in saline efflorescence (E) and had no quiescent microorganisms on the surface (Fig. 3B). The sample from area C observed by SEM showed little sign of salt and gypsum particles and the surface was fairly uniform, despite some cracks included saline efflorescence (E) (Fig. 3C).

3.2. Bacteria growing on endogenous carbon of fresco fragments

After 18 h incubation at 28 °C turbidity due to bacterial growth (LM observation) was only observed for samples from area A. No bacterial growth was evident for samples from areas B and C, even after 72 h of incubation.

To determine the new elemental composition of fragments from area A after culture in mineral medium, the fragment were again analysed by SEM/EDX (see Fig. 2 area A incubated). Surprisingly, an increase in total carbon from 18% to 37% was recorded. This significant increase may be due to active bacterial colonization of the fragments. There was also a simultaneous decrease in sulphur from 12.2% to 1.4% and complete disappearance of total calcium from the fragment surface. The latter two elements were presumably partly taken up as microelements by the microbes and/or went into solution. Other elements, such as potassium (7.5%), sodium (7.1%) and phosphorus (5.3%) were introduced with the mineral medium.

3.3. Molecular analysis

Two distinct strains were isolated from culture on complete solid medium on the basis of colony morphology.

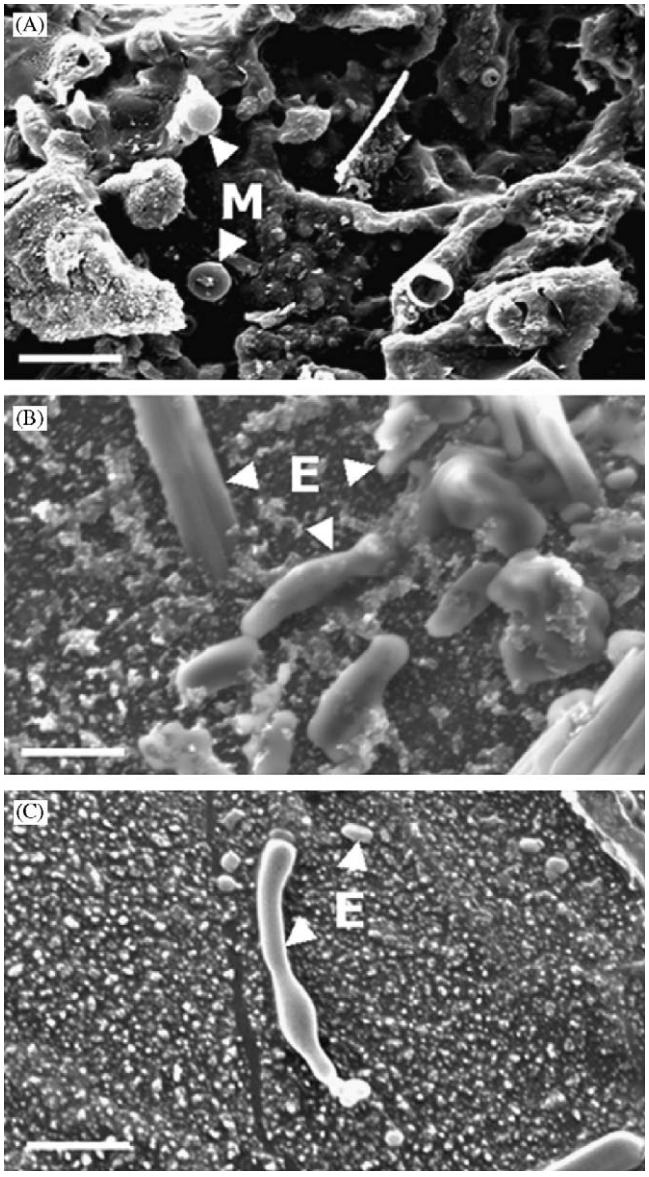

Fig. 3. SEM observation of original fresco fragments without incubation : (A) fresco surface with quiescent microbes (M) in site A (bar = 10 μm); (B) fresco surface with abundant saline efflorescence (E), in site B (bar = 5 μm); (C) chemical deterioration of fresco surface showing saline efflorescence (E) in site C (bar = 10 μm).

LM showed that CV1 had pink colonies with coccus-like elements forming dyads and tetrads that stained Gram positive. Strain CV2 had yellow colonies with pointed rod elements that stained Gram negative.

Using three universal bacterial primers we determined 1300 bases of the 16S rRNA gene sequence of strains CV1 and CV2. In order to investigate the phylogenetic relationship of the isolates to other bacteria, the sequences were aligned with those contained in public databases (GenBank). Comparative analysis revealed that strain CV1 (Gen Bank accession nos. DQ176452) belonged to the order Actinomycetales and was identified as *Kocuria erythromyxa* (formerly *Micrococcus roseus*) with 100% sequence homology to the reference strain. Strain CV2

(Gen Bank accession nos. DQ176453) belonged to the *Sphingomonadales* and was identified as *Sphingomonas echinoides* (formerly *Pseudomonas echinoides*) with 100% sequence homology.

3.4. Microscopic analyses of biofilm

To investigate the capacity of the strains to adhere to the surface of the fresco fragment in an attempt to explain the high total carbon content after incubation, we looked for interaction between the fresco surface and the microorganisms by CSLM. This nondestructive method can be used to observe biofilms in three dimensions in situ (Neu, 2000) during EPS formation (Wingender et al., 1999). Fluorescent labelling with site-specific lectins localises proteins that bind carbohydrates (Baldi et al., 2003). In this case, ConA incubated with ConA–FITC specifically bound residues of the polysaccharide containing glucose and mannose, and was used to detect adhesion of bacteria to the fresco fragments. We found extensive bacterial colonization of the fragments (Fig. 4A), and EPS that stained with ConA (Fig. 4B) indicating glucose and/or mannose residues (Baenzinger and Fiete, 1979).

Biofilm formation was confirmed by TEM analysis of cell–substrate interactions in fragments from area A of the fresco (Fig. 5). The only microorganisms from the cultured fragments in contact with the inorganic surface were typical cells of *S. echinoides* strain CV2. *K. erythromyxa* with its dyads and echinoid coccoid cells was not observed by TEM analysis. This probably means that the latter was not involved in biofilm formation, but we cannot exclude its presence in the biofilm as a minor species.

S. echinoides CV2, which has a typical pointed rod form adhered perfectly to the substrate (S) by means of very electron dense EPS (Fig. 6). The material was so electron negative that the cell walls were not well defined. This suggests that the EPS is secreted and is acid because it complexes electron dense metals. Many bacteria produce water-retained EPS to survive during drought (Morton and Surman, 1994; Beech, 2004). Prolonged humidity of the fresco due to bacterial biofilm would increase deterioration, magnify leaching from the fresco sublayers and accentuate sulphate accumulation on the fresco surface.

4. Conclusions

A major difficulty of this study was the small weight of samples. In situ techniques were therefore useful, because they only require small amounts of material. Areas A and B showed high total sulphur concentrations. Site A was the

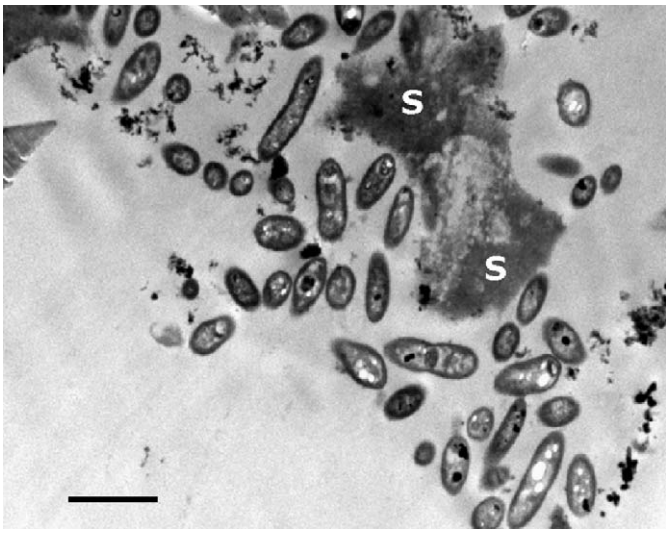

Fig. 5. TEM micrograph showing adhesion of strain CV2 to the fresco substrate (S). The strain of *S. echinoides* grew in mineral medium from fresco samples from area A (bar = 1.4 μm).

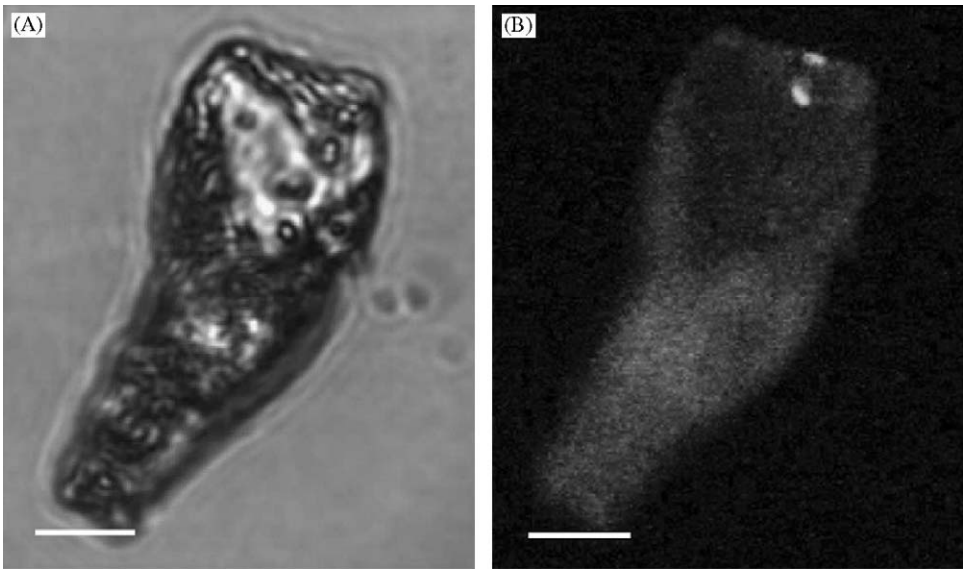

Fig. 4. (A) CSLM micrograph showing pointed rod elements of S. echinoides strain CV2 adhering to the surface of a sample; (B) ConA–FITC fluorescence of EPS containing glucose and mannose covering much of the surface of the sample (bar = 1 mm).

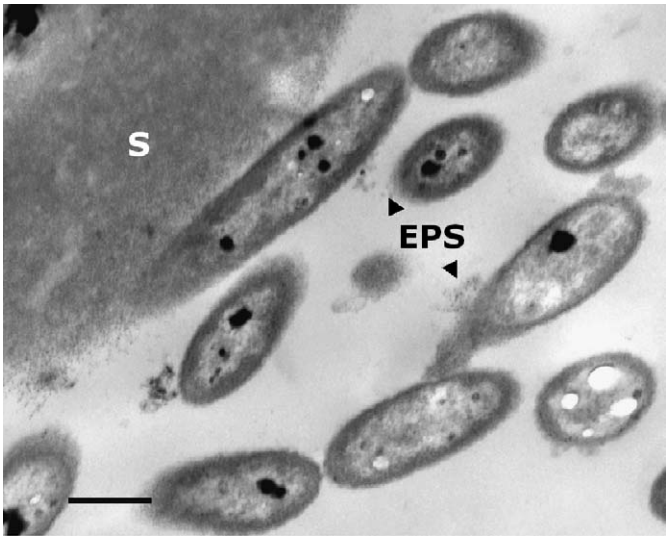

Fig. 6. TEM micrograph showing adhesion of strain to the fresco substrate (S) and EPS, (bar = 0.70 μm).

most damaged and had the highest total carbon content. The mixture of carbon compounds, originating from human and/or previous microbial activity, supported microbial growth of *Sphingomonas* and *Kokuria* strains. *S. echinoides* CV2 produced a moisture-retaining biofilm on the fresco surface. EPS of the biofilm may stimulate the growth of other microorganisms, increasing the damage. The deterioration in area A could therefore have been caused by rising damp from the floor combined with microbial activity.

Acknowledgements

The authors thank Director Prof. Anna Carli and Conservator Dr. Enrico Toti of the Museum complex Santa Maria della Scala, Siena, for encouragement and Mr. Fabrizio Iacopini for helping to obtain small fragments from the fresco.

References

Atlas, R.M., Chowdhury, A.N., Lal Gauri, K., 1988. Microbial calcification of gypsum-rock and sulfated marble. Studies in Conservation 33, 149–153.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 1994. Current protocols of Molecular Biology. Wiley, USA.

Baenzinger, J.U., Fiete, D., 1979. Structural determinants of Concanavalin A specificity for oligosaccharides. Journal of Biochemistry and Chemistry 254, 2400–2407.

Baldi, F., Pepi, M., Capone, A., Della Giampaola, C., Milanesi, C., Fani, R., Focarelli, R., 2003. Envelope glycosylation determined by lectins in microscopy sections of *Acinetobacter venetianus* induced by diesel fuel. Research in Microbiology 154, 417–424.

Beech, I.B., 2004. Corrosion of technical Materials in the presence of biofilms-current understanding and state-of-the art methods of study. International Biodeterioration & Biodegradation 53, 177–183.

Ciferri, O., 1999. Microbial degradation of paintings. Applied Environmental Microbiology 65, 879–885.

Eggins, H.O.W., Oxley, T.A., 2001. Biodeterioration and biodegradation. International Biodeterioration & Biodegradation 48, 12–15.

Giullitte, O., 1995. Bioreceptivity: a new concept for building ecology studies. The Science of the Total Environment 167, 215–220.

Gorbushina, A.A., Petersen, K., 2000. Distribution of microorganisms on ancient wall paintings as related to associated faunal elements. International Biodeterioration & Biodegradation 46, 277–284.

Gorbushina, A.A., Heyrman, J., Dornieden, T., Gonzalez-Delvalle, M., Krumbein, W.E., Laiz, L., Petersen, K., Saiz-Jimenez, C., Swings, J., 2004. Bacterial and fugal diversity and biodeterioration problems in mural painting environments of St. Martins church. International Biodeterioration & Biodegradation 53, 13–24.

Heyrman, J., Verbeeren, J., Schumann, P., Devos, J., Swing, J., De Vos, P., 2004. *Brevibacterium* picturae sp. Nov. Isolated from a damaged mural painting at the Saint-Catherine chapel (Castle Herberstein, Austria). International Journal of Systematic and Evolutionary Microbiology 54, 1537–1541.

Milanesi, C., Baldi, F., Vignani, R., Ciampolini, F., Faleri, C., Cresti, M., 2006. Fungal deterioration of medieval wall fresco determined by analysing small fragments containing copper. International Biodeterioration & Biodegradation 57, 7–13.

Morton, L.H.G., Surman, S.B., 1994. Biofilms in biodeterioration. International Biodeterioration & Biodegradation 34, 203–221.

Neu, T.R., 2000. In situ cell and glycoconjugate distribution of river snow as studies by confocal laser scanning microscopy. Aquatic Microbiology Ecology 21, 85–95.

Normal DL 3/80, 1980. Materiali lapidei, campionamento. CNR-ICR, Cosmas grafica, Roma.

Overmann, J., Gemerden, H., 2000. Microbial interactions involving sulfur bacteria: implications for the ecology and evolution of bacterial communities. FEMS Microbiology Review 24, 591–599.

Roldán, M., Clavero, E., Hernãndez-Mariné, M., 2003. Aerophytic biofilms in dim habitats. In: Salz Jimênez, C. (Ed.), Molecular Biology and Cultural Heritage, Proceedings of International Congress, Sevilla. Balkema Publishers, Lisse (NL), pp. 163–169.

Saarela, M., Alakomi, H.L., Siuhko, M.L., Maunuksela, L., Raaska, L., Mattila-Sandholm, T., 2004. Heterotrophic microorganisms in air and biofilm samples from roman catacomb, with special emphasis on actinobacteria and fungi. International Biodeterioration & Biodegradation 54, 27–37.

Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26, 31–43.

Strzelczyk, A.B., 1981. Stone. In: Rose, A.H. (Ed.), Microbial Deterioration. Academic Press, London, pp. 61–80.

Urzì, C., Realini, M., 1998. Color changes of Noto,s calcareous sandstone as related to its colonisation by microorganisms. International Biodeterioration & Biodegradation 42, 45–54.

Warscheid, TH., Braams, J., 2000. Biodeterioration of stone: a review. International Biodeterioration & Biodegradation 46 (4), 343–368.

Wingender, F., Neu, T.R., Flemming, H.C., 1999. Microbial Extracellular Polymeric Substances. Sprinter, Berlin.

Zanotti-Censoni, A.L., Mandrioli, P., 1979. Aerobiological investigation in Scrovegni Chapel (Padua, Italy). In: Proceedings of the third International Congress on Deterioration and Preservation of Stone, Venezia. Litografia la Photograph, Padova, pp. 699–703.