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ORIGINAL ARTICLE

Novel *Staphylococcus* sp. isolated from wall scrapings of a historical building in India

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Abstract Recently, there has been increased interest in the preservation of historic monuments, and considerable effort is being employed in developing new methods for controlling weathering, especially biologically influenced deterioration. Increasing our knowledge about the types of organisms growing on facades will help us to prevent undesirable growth on these structures. This report describes isolation and characterization of a halotolerant bacterium isolated from wall scrapings of a historical building. This organism grew well in SM basal salt medium and nitrogen-free semi-solid LGI medium, and tolerated (1) high temperature (up to 55°C), (2) high salt concentration (20%), and (3) a wide range of pH (5.0-12.0). On the basis of its morphological attributes, biochemical characteristics and partial sequencing of 16S rRNA, it belonged to the genus *Staphylococcus* with 99% identity to *Staphylococcus saprophyticus*.

Keywords Biofilms · Historical building · Osmolytes · Salt-tolerant · *Staphylococcus*

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Introduction

Preservation of archaic stone structures and old classical paintings poses challenges for scientists. Chemical composition of artwork, environmental conditions, air pollution, biological attack and natural ageing are some of the factors that cause biodeterioration. Microorganisms cause irreversible aesthetic and structural damage to any type of artwork, resulting into degradation of our cultural heritage with serious economic implications (Strzelczyk 1981; Griffin et al. 1991; Tiano 1993; Koestler et al. 1997). Aesthetic damage includes pigment discoloration, stain and formation of biofilms, while structural damage includes cracking and disintegration of paint layers, formation of paint blisters, and degradation of support polymers or of glue and binders resulting in detachment of paint layers from their support and also weathering of surface layers (Shirakawa et al. 2002). Although a plethora of reports are available on damage of paintings, stone structures and masonry, only in a few cases have attempts been made to understand the chemical modification brought about by microbial colonization (Ciferri 1999). The organisms associated with biodeterioration processes grow on buildings as biofilms. A biofilm contains active and dormant microorganisms and products of their metabolism, such as acids and polymeric materials, often polysaccharides. The polymeric material acts as a glue and traps dirt from the air, which in turn acts as a source of nutrition for microorganisms. The various organic materials of paints also represent a carbon source for practically all species of microorganisms and act as nutrient to stimulate microbial growth (Obidi et al. 2009). This seriously compromises the adhesion and durability of the paint as well as its decorative functions (Da Silva 2003). Environmental pollutants such as sulphur dioxide cause direct damage to frescos and provide substrates promoting

growth of aerobic and anaerobic sulfur-cycling bacteria, which in turn supply organic and inorganic nutrients to heterotrophic bacteria (Sorlini et al. 1987, Bock and Sand 1993). In most cases, the colonization begins with autotrophic nitrifying microorganisms such as algae and cyanobacteria; they establish a primary biofilm supporting the growth of heterotrophs including bacteria and fungi (Karpovich-Tate and Rebrikova 1990). Thus, the main microorganisms involved in this biodeterioration are bacteria, fungi, algae, cyanobacteria, and lichens. This variety in the microbial community indicates the complexity of the populations influencing deterioration.

Interactions between biotic and abiotic factors significantly affect the microbial growth. The most important typical characteristic of all microorganisms growing on facades is their ability to endure repeated cycles of drying and dehydration (Gaylarde and Morton 1999). Microorganisms adapt to these extreme periodic changes in environment using various strategies such as (1) sporulation, (2) high osmotolerance, (3) completion of life cycle in brief periods of high humidity, (4) formation of resting propagules, (5) pleomorphism, etc. (Gaylarde and Gaylarde 2005). Most of the organisms depend on retention of very large amounts of osmolytes to resist desiccation.

The microbial populations on paint must be able to cope with high temperature and humidity and are therefore dominated by extremophiles including halophilic archae (Saunders et al. 2000). Salt efflorescences are common on masonry and are known to be associated with halotolerant bacteria (Incerti et al. 1997), especially in dry and hot climates, because of their ability to survive the desiccation occurring on the surfaces. The occurrence of different halophilic and halotolerant fungi such as *Hortaea, Aureobasidium pullulans, Werenckii* and *Cladosporium* from different ecological niches has been reported earlier (Bravery 1988). However, there are very few reports on bacterial isolates. The aim of the present study was to isolate high salt-tolerant bacteria from the wall scrapings of a historic building in Pune, Maharashtra, India. The knowledge of microbial diversity in biofilms on historic monuments would also help to develop correct conservation and restoration strategies.

Materials and methods

Samples were collected from scraping of biofilms of an old building (more than 300 years old) in Pune (India). Sampling was carried out in sterile screw-capped glass bottles and transported to the laboratory.

Samples (100 mg) were suspended in 1 ml of sterile distilled water and were used for inoculation by spreading on SM basal medium (Loganathan and Nair 2004). The plates were incubated at 37°C for 48 h. The cultures obtained were transferred on nitrogen-free LGI medium (Cavalcante and Dobereiner 1988) and incubated at 37°C for 24 h. They were maintained on yeast extract mannitol agar (YEMA) medium (composition per litre: K_2HPO_4 0.5 g, $K_2SO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.1 g, mannitol 10.0 g, yeast extract 1.0 g, agar 20.0 g, Congo red 1.00% solution).

The cultures obtained on nitrogen-free LGI medium were further screened for their salt tolerance. The nutrient broth supplemented with various concentrations of NaCl (ranging from 1 to 25%) was used for inoculation (10^7 cells/ml). Incubation was carried at 37°C, 120 rpm for 24 h. The total viable counts (TVC) of all cultures were determined on nutrient agar. The nutrient broth supplemented with 0.5% NaCl was used as a control.

The isolate showing high salt tolerance (15% and above) was selected for morphological and biochemical characterization, using different sugars (xylose, glucose, fructose, sucrose, lactose, and maltose). It was also studied qualitatively for its ability to produce extracellular enzymes (amylase, urease, protease, gelatinase, and β-galactosidase).

The genomic DNA was isolated as described by Ausubel et al. (1987). The PCR assay was performed using Applied Biosystems, model 9800 with 1.5 μl of DNA extract in a total volume of 25 μl. The PCR master mixture contained 2.5 μl of 10X PCR reaction buffer (with 1.5 M $MgCl_2$), 2.5 μl of 2 mM dNTPs, 1.25 μl of 10 pm/μl of each oligonucleotide primers 27f (5′ CCAGAGTTTGATCG TGGCTCAG 3′), 1488R (5′ CGGTTACCTTGTTACGAC TTCACC 3′), 0.24 μl of Taq DNA polymerase and 15.76 μl of glass-distilled PCR water.

Initially denaturation accomplished at 94°C for 3 min. Thirty-five cycles of amplification consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension phase at 72°C for 10 min was performed. The PCR product was purified by PEG-NaCl method. The sample was mixed with 0.6 times volume of PEG-NaCl [20% PEG (MW 6000), 2.5 M NaCl] and incubated for 40 min at 37°C. The precipitate was collected by centrifugation at 3,800 rpm for 28 min. The pellet was washed with 70% ethanol, air dried and sequenced using a 96-well plate.

The sample was sequenced using a 96-well Applied Biosystems sequencing plate as per the manufacturer's instructions. The thermocycling for the sequencing reactions began with an initial denaturation at 94°C for 2 min, followed by 35 cycles of PCR consisting of denaturation at 94°C for 10 s, annealing at 50°C for 10 s, and extension at 68°C for 4 min. The samples were purified using standard protocols described by Applied Biosystems, Foster City, USA. To this, 10 μl of Hi-Di formamide was added and vortexed briefly. The DNA was denatured by incubating at 95°C for 3 min, kept on ice for 5-10 min, and was sequenced in a 3730 DNA

Table 1 Biochemical and physiological characteristics of the isolate

Characteristics		Characteristics	
Spores	–	Tolerance to pH 5.0-12.0	+
Catalase	+	Optimum pH	8.0
Hemolysis	+	Growth in salt at (%, w/v):	
β-galactosidase	+	2	+
Acid production from:		5	+
D-Xylose	+	7	+
D-Glucose	+	10	+
D-Fructose	+	15	+
Sucrose	+	20	+
D-Maltose	+	Growth at temperature (°C):	
Lactose	–	10	–
Hydrolysis of:		30	+
Casein	–	40	+
Gelatin	–	50	+
Starch	+	55	+
Urea	+	60	–
Utilization of:		70	–
Glycerol	+		
Citrate	–		
Nitrate	–		

+ Positive, – negative

analyzer (Applied Biosystems) following the manufacturer's instructions. 16S rRNA partial sequence (around 700 bases) of the isolate was compared with those available in Gen Bank+EMBL+DDBJ+PDB databases using BLASTN 2.2.17.

High salt-tolerant nitrogen-fixing culture was further studied for pH tolerance in nutrient broth adjusted to pH 5.0–12.0. Incubation was carried out at 37°C, 120 rpm for 24 h and cell growth determined by measuring absorbance at 660 nm.

The isolate was used to examine its temperature tolerance by inoculating equal volume of overnight grown culture (10^7 cells/ml) in nutrient broth and incubating at temperatures ranging from 10 to 70°C for 24 h, and TVC was determined on nutrient agar plate.

The culture was used to study growth curve characteristics in presence of high salt (20% NaCl) and nutrient broth containing 0.5% NaCl used as control. Growth was determined at intervals of 2 h for 30 h by measuring absorbance at 660 nm.

All the experiments were carried out using equal volume of overnight grown culture as inoculum (10^7 cell/ml)

Results and discussion

Samples of building scrapings were used to isolate salt-tolerant nitrogen-fixing microorganisms. Of the 10 isolates obtained on SM basal medium, only 5 isolates were found to grow on nitrogen-free medium.

However, one isolate was able to tolerate salt concentration up to 20% (3.5 M). Higher NaCl concentration beyond 20% was found to inhibit its growth (Table 1). More organisms grew at the lower salt concentration and diversity was higher. In the medium containing 15-20% salt, the colonies present were small suggesting that, although capable of survival, these organisms were not truly halophilic but merely halotolerant. Just one type of organism was able to grow at this salt concentration. Salt-tolerant bacteria have been detected by both culture and molecular biology techniques on European monuments (Heyrman and Swings 2001). This is in line with our present report. Kiel and Gaylarde (2007) reported halotolerant fungi for the first time on the historic building in Brazil.

The isolate was characterized morphologically, biochemically, and physiologically (Table 1). It was found to be Gram-positive spherical coccus that occurred singly, in pairs, in short chains and in irregular clusters. It was non-motile and non-spore forming. It produced extracellular amylase, urease, catalase, and beta-galactosidase in culture.

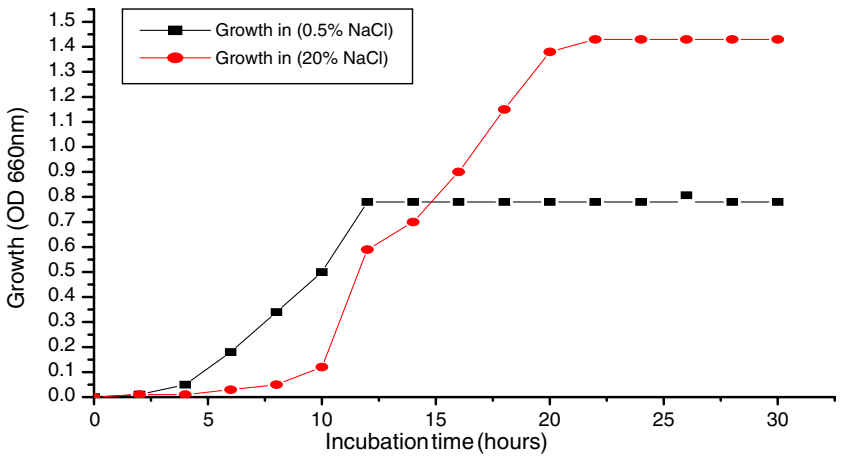

Fig. 1 Growth curve of *Staphylococcus saprophyticus* (this work). The cells were inoculated in nutrient broth containing 0.5 and 20% salt, respectively, and incubated at 37°C, 120 rpm. Their growth was determined by measuring absorbance at 660 nm

Acid was produced from D-xylose, D-glucose, D-fructose, and sucrose. It was also able to utilize citrate as carbon source. The characteristics indicated that it belonged to the genus S*taphylococcus* (Holt et al. 1994). This was also confirmed on the basis of comparative analysis of the 16S rRNA sequences. The BLAST search of partial 16S rRNA sequences of the isolate was carried out. The isolate matched best with the genus *Staphylococcus* and showed 99% similarity to *Staphylococcus saprophyticus* (accession no. DQ355389.1).

Staphylococcus saprophyticus (this work) grew well at a temperature of 55°C and tolerated a wide pH range of 5–12 with the optimum being 7.0–7.5 (Table 1). Growth curve studies indicated that the stationary phase occurred after 10 h in the presence of 0.5% NaCl (Fig. 1), while in the presence of 20% NaCl, a longer log phase was observed (Fig. 1). Many of the paint community, such as actinomycetes, gliding bacteria, aerobic spore-forming bacteria, and chlorophytes, form spores as an alternative survival strategy. Some bacteria commonly observed on walls may have other methods to resist desiccation, such as production of osmolytes, substances produced to protect cell materials against adverse conditions including desiccation and extreme temperatures. The main function of osmolytes is probably protection of protein conformation, preventing the denaturation of proteins (Saunders et al. 2000). They may also prevent the increase in conformational stability of nucleic acids induced by high salt concentration (Rajendrakumar et al. 1997). *Staphylococci* are known to adapt a second strategy to thrive in a low water activity niche. *Staphylococcus aureus* can grow in foods of water activity 0.86, equivalent to 3.5 $mols^{-1}$ NaCl (Scott 1953) due its ability to efficiently osmoregulate. It uses glycine, betaine, and proline as its osmolytes, which accumulate to a high intracellular concentration when the organism is subjected to high osmotic stress (Graham and Wilkinson 1992). A similar strategy may be adapted by *S. saprophyticus* isolated in this work.

This is probably the first report on a halotolerant *Staphylococcus* sp. with nitrogen fixation ability. *Staphylococcus* sp. isolated from rhizosphere of mangrove trees has been shown to increase nitrogen-fixation capacity of *Listonella anguillarnum* (Lindberg and Granhall 1984). A possible explanation may be the need for different bacteria to associate in order to create suitable conditions for nitrogen fixation. It has been expected that dinitrogen fixers grow best in presence of other heterotrophic bacteria which may stimulate nitrogen-fixers by physical and/or biochemical activities, thus receiving the required N compounds from the dinitrogen fixers (Tyler et al. 1979). Our isolate needs to be further characterized in mixed or pure culture for nitrogen-fixing ability, thereby proving its suitability as a soil inoculant.

Thus, *S. saprophyticus* isolated from wall scrapings of a historic building is a novel halotolerant organism with an ability to produce extracellular amylase and urease, high tolerance to a wide range of pH and temperature, and, most importantly, an unusual nitrogen-fixation ability.

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References

Ausubel FM, Rrent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) Current protocol in molecular biology. Wiley, New York

Bravery AF (1988) Biodeterioration of paint - a state-of-the-art comment. In: Houghton DR, Smith RN, Eggins HOW (eds) Biodeterioration. Elsevier, Barking, pp 466–485

Bock E, Sand W (1993) The microbiology of masonry biodeterioration. J Appl Bacteriol 74:503–514

Cavalcante VA, Dobereiner J (1988) A new acid-tolerant nitrogen fixing bacterium associated with sugar cane. Plant Soil 108:23–31

Ciferri O (1999) Microbial degradation of paintings. Appl Environ Microb 65:879–885

Da Silva VQ (2003) Microbial deterioration of paints. Microbiologist 4:43

Gaylarde CC, Gaylarde PM (2005) A comparative study of the major microbial biomass of biofilms on exterior of buildings in Europe and Latin America. Int Biodeterior Biodegrad 55:131–139

Gaylarde CC, Morton LHG (1999) Deteriogenic biofilms on buildings and their control: a review. Biofouling 14:59–74

Graham J, Wilkinson BJ (1992) *Staphylococcus aureus* osmo- for their possible roles in osmoregulation: role of choline, glycine betaine, proline and taurine. J Bacteriol 174:2711–2716

Griffin PS, Indictor N, Koestler RJ (1991) The biodeterioration of stone: a review of biodeterioration mechanisms, conservation case histories, and treatment. Int Biodeterior 28:187–207

Heyrman J, Swings J (2001) 16S rRNA Sequence analysis of bacterial isolates from biodeteriorated mural paintings in the Servilia tomb (Necropolis of Carmona, Seville, Spain). Syst Appl Microb 24:417–422

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST, Eds (1994) Bergey's manual of determinative bacteriology, 9th edn., Williams and Wilkins, USA

Incerti C, Blanco-Varela MT, Puertas F, Saiz-Jimenez C (1997) Halotolerant and halophilic bacteria associated to efflorescences in Jerez cathedral. In: Zezza F., Ed., Origin, Mechanisms and Ejects of Salts on Degradation of Monuments in Marine and Continental Environments. Protection and Conservation of the European Cultural Heritage Research, Report No. 4, pp. 225–232

Karpovich-Tate N, Rebrikova NL (1990) Microbial communities on damaged frescoes and building materials in the Cathedral of the Nativity of the Virgin in the Panfnutii-Borovskii Masonry. Russia Int Biodeterior 27:281–296

Kiel G, Gaylarde C (2007) Diversity of salt-tolerant culturable aerobic microorganisms on historic buildings in Southern Brazil. WJ Microbiol Biotechnol 23:363–366

Koestler RJ, Warscheid T, Nieto F (1997) Biodeterioration: risk factors and their management. In: Baer NS, Snegthlage R (eds) Saving our architectural heritage: the conservation of historic stone structures. Wiley, London, pp 25–36

Lindberg T, Granhall U (1984) Isolation and characterization of dinitrogen-fixing bacteria from the rhizosphere of temperate cereals and forage grasses. Appl Environ Microbiol 48:683–689

Loganathan P, Nair S (2004) *Swaminathania salitolerants* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate-solubilizing bacterium from wild rice (*Proteresia corctata* Tateoka). Int J Syst Evol Microbiol 54:1185–1190

Obidi OF, Aboaba OO, Makanjuola MS, Nwachukwu SCU (2009) Microbial evaluation and deterioration of paints and painted-products. J Environ Biol 30:835–840

Rajendrakumar CS, Suryanarayana T, Reddy A (1997) DNA helix destabilization by proline and betaine: possible role in the salinity tolerance process. FEBS Microbiol Lett 410:201–205

Saunders AJ, Davis-Searles PR, Allen DL, Pielak GJ, Erie DA (2000) Osmolyte-induced changes in protein conformational equilibria. Biopolymers 53:293–307

Scott WJ (1953) Water relations of *Staphylococcus aureus* at 30°C. Aust J Biol Sci 6:549–564

Shirakawa MA, Gaylarde CC, Gaylarde PM, Jonh V, Gambale W (2002) Fungal colonization and succession on newly painted buildings and the effect of biocide. FEMS Microbiol Ecol 39:165–173

Sorlini C, Sacchi M, Ferrari A (1987) Microbiological deterioration of Gambara's frescos exposed to open air in Brescia. Italy Int Biodeterior 23:167–179

Strzelczyk AB (1981) Paintings and sculptures. In: Rose AH (ed) Microbial deterioration. Academic, London, pp 203–234

Tiano P (1993) Biodeterioration of stone monuments: a critical review. In: Garg KL, Garg N, Mukerji KG (eds) Recent advances in biodeterioration and biodegradation, Vol. 1. Naya Prokash, Calcutta, pp 301–321

Tyler ME, Milam JR, Smith RL, Schank SC, Zuberer DA (1979) Isolation of *Azospirillum* from diverse geographic regions. Can J Microbiol 25:693–697