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Molecular characterisation of *Halobacillus* strains isolated from different medieval wall paintings and building materials in Austria

Katrin Ripka[a], Ewald B.M. Denner[b], Astrid Michaelsen[a], Werner Lubitz[a], Guadalupe Piñar[a,*]

[a]*Department of Medical/Pharmaceutical Chemistry, University of Vienna, UZAII, 2B522, Althanstr, 14, A-1090 Vienna, Austria*
[b]*Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine Vienna, Veterinärplatz 1, A-1210 Vienna, Austria*

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Abstract

We previously reported on the detection and isolation of an indigenous population of *Halobacillus* from salt-damaged medieval wall paintings and building materials of Herberstein castle in St. Johann bei Herberstein in Styria, Austria. Several moderately halophilic, Gram-positive, endospore-forming *Halobacillus*-like bacteria could be again isolated by conventional enrichment from salt efflorescences collected in the medieval St. Virgil's chapel in Vienna. Comparative 16S rDNA sequence analyses showed that the St. Virgil isolates are most closely related (>98.5% sequence similarity) to *Halobacillus trueperi*, *Halobacillus litoralis,* and to our previous halobacilli strains obtained from the castle Herberstein. Based on 16S rDNA sequence analysis, the strains could be clustered in three different groups. Group I: St. Virgil strains S3, S4, S21, and S22 (99.8–100% sequence similarity); group II: Herberstein strains K3-1, I7, and the St. Virgil strain S20 (99.3–99.7% sequence similarity); and group III: Herberstein strains I3, I3A, and I3R (100% sequence similarity). Molecular typing by denaturing gradient gel electrophoresis (DGGE), random amplified polymorphic DNA (RAPD-PCR), and internal transcribed spacer-homoduplex–heteroduplex polymorphism (ITS-HHP) fingerprinting showed that all isolates are typeable by each of the methods. RAPD was the most discriminatory method. With respect to their physiological characteristics—i.e., growth in the presence of 5–20% (w/v) NaCl, no growth in the absence of NaCl, optimum growth at 37 °C in media containing 5–10% (w/v) NaCl, and optimum pH around 7.5–8.0—the St. Virgil isolates resembled our previously isolated strains. However, the St. Virgil strains showed some differences in their biochemical properties. St. Virgil isolates hydrolysed Tween 80, two isolates reduced nitrate, and no isolate liquefied gelatine. The recurrent isolation of halobacilli from salt efflorescences on historic buildings and monuments at two different geographical locations may indicate that this group of bacteria is common in salt-affected ruins.

Keywords: Wall paintings; *Halobacillus* sp.; DGGE; RAPD-PCR; ITS-HHP

1. Introduction

Microorganisms play a crucial role in the deterioration processes of building materials and objects of art, particularly if such materials are exposed to the open air (Bock and Sand, 1993; Ciferri, 1999; Herrera and Videla, 2004; Peraza-Zurita et al., 2005). Knowledge about the complexity and diversity of the bacterial communities present in such habitats has increased enormously in recent years because of the use of molecular techniques such as denaturing gradient gel electrophoresis (DGGE) analysis (Rölleke et al., 1998; Gurtner et al., 2000; Gonzalez and Saiz-Jimenez, 2005). The information revealed by analyses of 16S rDNA sequences directly obtained from amplified environmental DNA allows the detection of bacteria not yet cultivated. These data can be then used to design specific culture media for the cultivation of the previously detected microorganisms.

Several investigations based on culture-independent (Rölleke et al., 1998; Piñar et al., 2001a, b) as well as on culture-dependent techniques (Heyrman et al., 1999; Laiz et al., 2000; Saiz-Jimenez and Laiz, 2000) have demonstrated that monuments where salt efflorescences on the

*Corresponding author. Tel.: +43 1 4277 55116; fax: +43 1 4277 55120.
E-mail address: guadalupe.pinar@univie.ac.at (G. Piñar).

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surfaces are a common phenomenon, as a result of changing physical parameters, may be a habitat for extremely salt-tolerant and moderately halophilic microorganisms. The molecular detection and isolation of *Halobacillus* species in deteriorated wall paintings and building materials of the Catherine chapel at Herberstein castle (Styria, Austria) (Piñar et al., 2001c) prompted further studies to focus on this group of bacteria not previously reported from stoneworks. In the past, halobacilli were overlooked due to the use of unsuitable culture media with inadequate salt concentration, and/or the long incubation time required for the growth of this group of bacteria under laboratory conditions.

Recently, we cultivated *Halobacillus*-like bacteria from stonework samples of a medieval building in Austria (St. Virgil's chapel, Vienna). Here we report on the molecular characterisation of *Halobacillus* strains from wall paintings and building materials.

2. Materials and methods

2.1. Description of the site and sampling

This study was carried out in two different locations, the Catherine chapel at Herberstein castle (Styria, Austria) and the St. Virgil Chapel (Vienna, Austria). The Catherine chapel at Herberstein castle is decorated with several frescoes from the 14th century. The wall paintings were discovered and partly exposed around 1930, but restoration did not start before the 1940s. However, after restoration, the chapel was neglected and used as storage room until the mid-90s. The location is exposed to humidity fluctuation at irregular intervals. Two samples were collected from different areas of the wall paintings: sample H1 was taken below the chancel east wall window where a brownish biofilm was observed; sample H6 was taken from the chancel north wall, from a zone of the painting with an intense rosy discoloration. An additional sample (m7) was taken outside the chapel, from a limestone wall also showing an intense rosy discoloration.

The St. Virgil Chapel dates back to the beginning of the 14th century. It is located under the ruins of St. Mary Magdaleine Chapel. St. Virgil Chapel was originally created as a tomb. After the Chapel of St. Mary Magdaleine was destroyed in 1781 by a fire and demolished, the underground room was abandoned. The chapel was rediscovered during an excavation in 1972. The rectangular room is 10.5 m long and 6 m wide. The mortar and rubble walls are 1.5 m thick, containing six recesses with pointed arches, one of which was removed to open the present-day entrance. They are decorated with large red crosses painted onto white plaster. The whole chapel walls are encrusted with salt efflorescences visible to the naked eye. A sample (S3) was directly taken from a salt efflorescence.

All samples were collected with sterile scalpels and vials by scraping off surface material and plaster to a depth of 1–3 mm. Samples were used within a few hours for conventional enrichment and cultivation.

2.2. Cultivation strategy

Two different media were used for enrichment: M2 medium (Tomlinson and Hochstein, 1976) and maintenance medium (MM) (Spring et al., 1996). Enrichments were conducted in 300 mL Erlenmeyer flasks containing 30 mL medium. To avoid fungal growth, media were supplemented with 50 $\mu g\,mL^{-1}$ cycloheximide (Sigma). Flasks were incubated aerobically at room temperature (22 ± 3 °C) and at 37 °C in a water bath by shaking using magnetic stirring bars. Over a total period of 3 weeks, weekly aliquots of 100 µL enrichments were plated onto four different solid media: M2 medium (20% w/v NaCl), M2A medium (20% w/v NaCl) (Denner et al., 1994), Halococci medium (25% w/v NaCl), and LB medium (Atlas, 1995). All media were incubated aerobically at room temperature and at 37 °C.

2.3. Bacterial strains

Strains K3-1, I3, and I7 (Piñar et al., 2001c), and strains I3R and I3A were isolated from the chapel of Herberstein castle. Strains S3, S4, S20, S21, and S22 were isolated from the stonework of the chapel of St.Virgil in Vienna. All isolates were stored at −70 °C in MM broth containing 20% (w/v) glycerol as cryoprotectant. Bacterial inocula for all experiments were taken from cryopreserves, streaked onto MM plates and cultivated at 37 °C for 48 h. Reference type strains of *Halobacillus* used in this study were: *Halobacillus karajensis* DSM 14948^T, *Halobacillus halophilus* DSM 2266^T, *Halobacillus litoralis* DSM 10405^T, *Halobacillus trueperi* DSM 10404^T, *Halobacillus locisalis* DSM 16468^T (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and *H. salinus* JCM 11546^T (JCM, Japanese Collection of Microorganisms, RIKEN BioResource Center, Japan).

2.4. Phenotypic characterisation

Growth at different NaCl concentrations (0%, 1%, 5%, 10%, 15%, 20%, 25%, and 30% w/v) was determined on M2A agar (Denner et al., 1994); plates were incubated aerobically at 37 °C for 48 h. Cell morphology was examined on a Leitz Diaplan (Germany) phase contrast microscope. Gram staining, KOH-lysis test and conventional biochemical tests (i.e., catalase, nitrate reduction, oxidative-fermentative metabolism of glucose [O/F test], citrate utilisation, indole production, hydrolysis of casein and starch) were carried out as described by Smibert and Krieg (1994). Oxidase activity was tested using commercial test strips (Bactident oxidase; Merck).

2.5. Molecular characterisation

2.5.1. DNA extraction and PCR analysis

Genomic DNAs were extracted according to the protocol provided by Ausubel et al. (1991). For PCR analysis, 2 × PCR Master Mix (Promega) (50 units/mL of *Taq* DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$) was diluted to 1 × and 12.5 pmol of each primer were added. PCR was carried out in 25 µL volumes and 2.5 µL template was added.

Two different PCR reactions were carried out to amplify eubacterial 16S rDNA fragments. For DGGE analysis, 200-bp fragments of the 16S rDNA were amplified using the eubacterial specific primer 341fGC (forward) to which a 40-base GC clamp was added to its 5′ end (Muyzer et al., 1993). As reverse primer, the universal consensus primer 518r (Muyzer et al., 1993) was used. For sequencing analyses, ~1500-bp 16S rDNA fragments were amplified using the forward primer 27f and the reverse primer 1492r (Lane, 1991). PCR reactions were performed using a Robocycler (Stratagene). PCR conditions: 5 min denaturation (95 °C), followed by 30 cycles consisting of 1 min denaturation (95 °C), 1 min primer annealing (55 °C) and 2 min primer extension (72 °C), with a final extension step of 72 °C for 5 min.

2.5.2. DGGE

DGGE was done as previously described (Muyzer et al., 1993) using a D GENE system (Bio-Rad) in 0.5 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8). Conditions used: lineal chemical gradient ranged from 25% to 60% (100% denaturant contains 7 M urea and 40% v/v formamide). Gel electrophoretic separation was done at 60 °C and 200 V for 3.5 h. Subsequently, gels were stained with ethidium bromide and documented using a UVP gel documentation system.