Microbiological survey of the stone chambers of Takamatsuzuka and Kitora tumuli, Nara Prefecture, Japan: a milestone in elucidating the cause of biodeterioration of mural paintings

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Introduction

The Takamatsuzuka tumulus (TT) and the Kitora tumulus (KT), circular burial mounds located in Asuka village, Nara Prefecture, Japan, are thought to have been built in the late 7th to early 8th centuries and were designated Special Historic Sites by the government in 1973 and 2000, respectively. The colorful mural paintings covering the interior walls and ceilings of the stone chambers have suffered more damage in the past 35 years than in the previous 1,300 years.

TT was excavated in 1972. The interior of the chamber is 2.7 m deep, 1.0 m wide, and 1.1 m high (Fig.2, on P.44 in this book). The ceiling mural depicts star constellations (Seishuku), while the wall murals portray the sun and moon (Jitsugetsu), the four heavenly guardian gods (Shijin), and groups of men and women. In particular, the group of four women on the west wall is commonly called the “Asuka beauties” by the press (Fig.1, on p.43 in this book). All the paintings were designated as National Treasures by the government in 1974. In accordance with specialists’ advice, the Japanese Agency for Cultural Affairs decided in 1973 to preserve the mural paintings in the chamber interior, which has never been opened to the public. It was thought that the mural paintings’ beauty and stability could be preserved if high humidity (100%RH) and temperature (14-20°C) could be maintained underground, as these had been the conditions in the natural unexcavated state. In addition, the chamber has been kept in darkness except for regular inspections by the conservation staff. The lack of light and the other conditions were initially thought to be a nutrient-poor environment that would inhibit the growth of microorganisms and micro-animals, which could deteriorate the murals. The major records on microbiological inspections since April 1972 are outlined in Table 1, which summarizes the history of the effort to save the mural paintings and the chamber interior (cf. Kigawa et al. in this book). Particularly after remediation work at space adjacent to the chamber in spring 2001, fungi again grew on the 1,300-year-old murals, causing serious deterioration (Fig.4 on p.46, Fig.5 on p.47 in this book). In addition, viscous gels called biofilms (e.g., de Beer and Stoodley 2006), samples of which frequently contained mites that were presumably feeding on the molds and yeasts, were also visible on the wall plaster and floor after 2004. Because of the state of deterioration, a big project to relocate the chamber began in April 2007. The chamber was dismantled by a team organized by the Agency. By mid-2007, all the colorfully painted stone walls were restored at an outside facility in Asuka village to save them from further deterioration and for necessary restoration.

KT, on the other hand, was excavated in early 2004. The stone chamber is very similar to that of TT in size as well as in the characteristics of the mural paintings. The star chart on the chamber ceiling is the oldest example of star constellations in East Asia, and all four guardian gods—the Blue Dragon (Seiryu), White Tiger (Byakko), Black Snake-tortoise,
The environmental conditions in the chamber are quite similar to those in TT, but the paintings on plaster are partly detached from the wall as support. Considering the serious conditions inside the chamber, the Agency decided in July 2004 to move these paintings to a controlled environment, and by February 2007 all but the star chart on the ceiling had been relocated. An outline of the results of microbiological inspections since summer 2003 and of notable related information is also summarized in Table 2.

In elucidating the cause of biodeterioration of the mural paintings, we have been conducting microbiological surveys inside the TT and KT chambers since May 2004. We employ molecular biological methods in addition to conventional approaches of sample collection, isolation, and phenotypic characterization in order to elucidate the microbiota, the identities of the microorganisms, and the causes of biodeterioration. This article represents the outline of the data we have obtained thus far.

Table 1  A brief outline of the selected records of microbiological inspections in the TT stone chamber since the discovery*

<table>
<thead>
<tr>
<th>Date</th>
<th>Events / actions</th>
<th>Dominant microorganisms**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1972</td>
<td>Discovery</td>
<td>Alternaria, Cladosporium, Nigrospora, Trichoderma viride</td>
</tr>
<tr>
<td>1975</td>
<td>Excavation and the 1st microbiological inspection</td>
<td>Doratomyces, Fusarium, Cladosporium, Mucor</td>
</tr>
<tr>
<td>1976</td>
<td>Microbiological survey</td>
<td></td>
</tr>
<tr>
<td>1976-early 1980s</td>
<td>Periodic intense restoration</td>
<td>Doratomyces, Streptomyces</td>
</tr>
<tr>
<td>1980-1981</td>
<td>Fungal outbreak</td>
<td></td>
</tr>
<tr>
<td>1982</td>
<td>Stability and balance were gradually recovered</td>
<td>Aspergillus, Fusarium, Trichoderma, bacteria (actinomycetes)</td>
</tr>
<tr>
<td>1986-1987</td>
<td></td>
<td>Penicillium, Aspergillus, Fusarium, Trichoderma</td>
</tr>
<tr>
<td>Spring 2001</td>
<td>The remediation work at the adjacent space</td>
<td>Penicillium, Aspergillus, Fusarium, Cladosporium,</td>
</tr>
<tr>
<td>2002</td>
<td>May 2004 Viscous gels (biofilms) were visible on the wall plaster and floor</td>
<td>Cylindrocarpon, Gliomastix(sic), Trichoderma</td>
</tr>
<tr>
<td>Sep 2005</td>
<td>Cooling of the burial mound started</td>
<td>Penicillium, Fusarium, Trichoderma, Candida</td>
</tr>
<tr>
<td>Feb 2006</td>
<td>Outbreak of black stains / spots on the Asuka beauties</td>
<td>Bacillus, Ochrobactrum, Stenotrophomonas</td>
</tr>
<tr>
<td>May-Dec 2006</td>
<td>Outbreak of black stains / spots on the murals</td>
<td></td>
</tr>
<tr>
<td>Apr-Aug 2007</td>
<td>Dismantling and relocation of the stone chamber</td>
<td>Acremonium (sect. Gliomastix), Penicillium</td>
</tr>
</tbody>
</table>


** Generic names in bold show the major colonizers.

Table 2  A brief outline of the selected records of microbiological inspections in the KT since the discovery*

<table>
<thead>
<tr>
<th>Date</th>
<th>Events / actions</th>
<th>Dominant microorganisms**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1983</td>
<td>Discovery</td>
<td>Aspergillus, Fusarium, Penicillium, Trichoderma, Cunninghamella</td>
</tr>
<tr>
<td>2003</td>
<td>Fungal appearances in the adjacent small room</td>
<td>Acremonium, Penicillium, Trichoderma</td>
</tr>
<tr>
<td>Mar-July 2004</td>
<td>Excavation</td>
<td>Phialocephala</td>
</tr>
<tr>
<td>Aug 2004</td>
<td>Fungal colonies were found on the murals</td>
<td>Fusarium, Penicillium, Trichoderma</td>
</tr>
<tr>
<td>Sep 2004</td>
<td>Outbreak of needle-like fruiting bodies on the stone walls in the adjacent small room</td>
<td>Cylindrocarpon, Aspergillus</td>
</tr>
<tr>
<td>Oct-Dec 2004</td>
<td>The Agency decided to relocate the murals to a controlled environment</td>
<td>Bacillus megaterium</td>
</tr>
<tr>
<td>2005</td>
<td>Viscous gels (biofilms) were visible on the wall plaster and floor</td>
<td>Penicillium, Fusarium, Trichoderma, Cylindrocarpon</td>
</tr>
<tr>
<td>Sep 2005</td>
<td>Holes on the plaster walls were seen</td>
<td>Acremonium, Phialocephala, Candida, Stenotrophomonas, Serratia, Sphingomonas, Pseudomonas</td>
</tr>
<tr>
<td>Apr 2006</td>
<td>Outbreak of black particles on the ceiling</td>
<td>Gluconacetobacter</td>
</tr>
<tr>
<td>Oct 2006</td>
<td>Outbreak of black spots / stains on the walls</td>
<td>Burgia, Penicillium</td>
</tr>
<tr>
<td>Feb 2007</td>
<td>All paintings, except the star chart on the ceiling, were relocated</td>
<td>Acremonium (sect. Gliomastix), Penicillium</td>
</tr>
<tr>
<td>May 2007</td>
<td>Further outbreak of black spots / stains on the walls</td>
<td></td>
</tr>
</tbody>
</table>


** Generic names in bold show the major colonizers.
Materials and methods adopted in this study

To address the biodeterioration problems of the murals of both tumuli, it is essential to know the features of the substrates (e.g., plaster wall, stone wall), environmental factors, and microbial diversity, as well as to accurately identify, bioprofile, and characterize the microorganisms. Past papers provide numerous findings (e.g., Guglielminetti et al. 1994, Garg et al. 1995), as does Ciferri’s review (1999).

We collected a total of 663 samples from TT between May 2004 and August 2007. These are mainly cotton swab samples of moldy spots and biofilms. When the stone chamber was dismantled, we collected soils, plaster fragments, and plant roots. Fig.1 illustrates the methods we used, from sample collection to the identification of microbial isolates. From samples collected by sterilized cotton swabs, the microorganisms were isolated by smear plating (for bacteria and fungi) and placement in a moist chamber (for fungi; Gams et al. 1987, Krug 2004). Cultural and morphological data were obtained from each pure isolate. At the same time, the collected samples were carefully observed under both stereo- and light microscopes; the data were compared with those from the isolation. For the selected isolates, we determined gene sequences that are widely used for molecular phylogenetic analyses. Molecular trees were reconstructed using the neighbor-joining (NJ), maximum parsimony (MP), and Bayes methods; each method is shown in a figure. Statistical support values in the NJ tree were computed by a bootstrap analysis performed with 1000 replicates, whereas the support of nodes in the Bayesian tree was measured by posterior probabilities obtained from the majority rule consensus (for details, see Kiyuna et al. 2008).

To identify microbial isolates, we adopted an integrated analysis of both phenotypic and genotypic characteristics. For samples that could not be easily cultured, we directly isolated the DNA and then applied denaturing gradient gel electrophoresis (DGGE) analysis to obtain a DNA band for determination of the sequences before comparative analyses. DGGE is now applied to the detection of particularly dominant members in the microbial community without cultivation (for bacteria, see

Changes in mycobiota and the major colonizers

A total 107 samples were collected from the TT stone chamber interior from May 2004 through December 2006, and 454 fungal strains were isolated. Fig. 2 shows the changes in the makeup of the mycobiota over time, along with the numbers of samples and isolates. Between May and September 2004, \textit{Fusarium}, \textit{Trichoderma}, and \textit{Penicillium} were the predominant fungal genera. These genera are anamorphic fungi, which produce large quantities of conidia as asexual spores from the respective phialides. The following September, cooling down of the mound was started before the chamber was dismantled. Since then, \textit{Fusarium} and \textit{Trichoderma} in particular have decreased; after May 2006, they were replaced with the frequent growth of dark \textit{Acremonium}, i.e., the section \textit{Gliomastix} of the genus \textit{Acremonium}, considered to be the cause of the dark stains around the Asuka beauties on the west wall. \textit{Fusarium} and \textit{Trichoderma} are identified as the predominant fungi inside the chamber, together with the constant colonizers \textit{Penicillium} and yeasts, which were discovered in all samples throughout the period.

On the other hand, a total of 151 samples were collected and 534 fungal isolates were obtained from inside KT’s chamber and a small adjacent room from June 2004 through September 2007 (data not shown). Members of \textit{Penicillium}, \textit{Acremonium} (sect. \textit{Gliomastix}), \textit{Fusarium}, and \textit{Trichoderma} were isolated as the major fungal colonizers. Yeasts were also isolated throughout the period. Two noteworthy fungi—the strictly anamorph fungus \textit{Phialocephala} sp. and the basidiomycete anamorph \textit{Burgoa} sp.—appeared in KT on a wall of the small room and on the ceiling of chamber interior, respectively. The details are mentioned later.

Identity and molecular phylogenetic diversity of the major fungal colonizers

Figures 3-6 illustrate the molecular phylogenetic assignments and parts of the bioprofiles of the fungi isolated as the predominant colonizers in TT and KT. These are \textit{Fusarium}, \textit{Trichoderma}, \textit{Penicillium}, and
Acremonium (sect. Gliomastix). Except for *Penicillium*, these are the representative phialidic anamorph genera of the ascomycete order Hypocreales (Seifert and Gams 2001). Among these three genera, *Fusarium* is the most species-rich genus within the order that accommodates the phialidic anamorph genera *Cylindrocarpon*, *Gliocladium*, *Verticillium*, and others. In general, Hypocrealean anamorphs produce nondemataceous conidia in slimy masses from phialidic conidiogenous cells and chlamydospores as thick-walled resting structures. On the other hand, *Penicillium* is the most species-rich anamorph genus within Trichocomaceae, Eurotiales, Ascomycota. Molecular phylogenetic diversity and related problems with *Fusarium* and *Trichoderma* isolates from both tumuli have been fully discussed in Kiyuna et al. (2008).

*Fusarium* is a phialidic, macroconidial genus and is associated with perithecial teleomorphs of Nectriaceae, Hypocreales, Ascomycota (e.g. Rossman et al. 1999). These fungi are soil-inhabiting, lignicolous, and herbicolous (Domsch et al. 2007). *Fusarium* species are characterized by fusoid, transverse septate macroconidia with a basal contracted foot cell. Microconidia, also produced from phialides, develop in some species (cf. Fig. 3).

We estimated the phylogeny of 24 TT and KT *Fusarium* isolates using a translation elongation factor 1-α gene (*tef1*). As shown in Fig. 3, all isolates were placed in three clades: the *Fusarium solani* species complex (FSSC; O’Donnell 2000, O’Donnell et al. 2007), the *F. avenaceum/F. tricinctum* species complex (FA/FTSC; Yli-Mattila et al. 2002, Kiyuna et al. 2008), and the *F. oxysporum* species complex (FOSC; O’Donnell et al. 1998). Twenty-one TT and KT isolates were placed in clade 3 of the FSSC, thus...

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**Fig. 3** Cultural and morphological characteristics, and molecular phylogenetic assignment of *Fusarium* isolates from both tumuli. Abbreviations: T, Takamatsuzuka tumulus; K, Kitora tumulus; LSP 1-LSP 3, three phylogenetic species of clade 3 of the FSSC (Dupont et al. 2007); red characters denote isolates from the TT or KT interior; black characters denote isolates from the adjacent space or small room of either tumulus. Numbers at the selected branches are percent bootstrap values/Bayesian posterior probabilities; for details, see Kiyuna et al. (2008). Bar = 0.05 substitution/site.
suggesting haplotype (haploid genotype) diversity. The remaining one and two KT isolates were accommodated in the FOSC and FA/FTSC, respectively. FSSC clade 3 accommodated two notable isolates. One strain, TBT-3, was isolated from a sample taken in December 2001 from the north wall of the TT chamber interior, and produced a whitish colony. A similar colony was produced by TBT-4, which was isolated from a sample taken in October 2002 from a blackish, deteriorated part near the blue dragon on the east wall of the TT chamber. Our molecular phylogenetic analyses (Fig. 3) suggested that most TT isolates placed within clade 3 colonized in the chamber interior after the remediation work in the adjacent space was done in spring 2001. As shown in blue characters in the tree, the tef1 phylogeny included seven strains assignable to three phylogenetic species–LSP1, LSP2, and LSP3–within FSSC clade 3, all three of which had been collected from the Lascaux cave in France (Dupont et al. 2007). Fusaria from TT, KT, and the Lascaux cave were clustered in clade 3, but none of the fusaria from these three sites were clustered in clade 1 or 2 of the FSSC. Members of FSSC clade 3 are well known as plant-pathogenic, saprobic (mainly in soil), and mycotoxinogenic. Recently, O’Donnell et al. (2004, 2007) and Zhang et al. (2006) reported that fusaria pathogenic to humans and other animals are nested within either the FSSC or the FOSC. In light of Dupont et al.’s (l.c.) conclusions on Lascaux cave’s fusaria, we suggest that most TT and KT fusaria of FSSC clade 3 were also deeply related to the causes of deterioration of the murals. The taxonomy of the FSSC is still unresolved (O’Donnell 2000). We are carefully investigating the invasion roots and association between fusaria as a major fungal colonizer and the biodeterioration of the murals and walls of TT and KT.

**Trichoderma**

*Trichoderma* is a well-known, ubiquitous soil fungus having cellulolytic abilities (Domsch et al. 1993).
It is characterized by repeatedly branched conidiophores in tufts with divergent, often irregularly bent, flask-shaped phialides, from which usually green ameroconidia in slimy masses are produced (cf. Fig. 4), and is associated with perithecial telemorphs of Hypocreia (Rossman et al. 1999, Chaverri and Samuels 2003, Samuels 2006).

A total of 18 Trichoderma strains were isolated from the inside and outside of the TT and KT chambers, and were molecular-phylogenetically analyzed using the same gene, tef1. As shown in the tef1 phylogeny (Fig. 4), these isolates were assignable to the Harzianum-Virens clade (nine TT and four KT isolates), the Viride clade (four KT isolates) and Trichoderma section Longibrachiatum (one TK isolate). Also placed in the Harzianum-Virens clade were two isolates–TBT-5, derived from a moldy colony on treated soil in the space adjacent to the TT chamber (collected September 2003) and TBT-7, derived from a moldy colony on the upper part of a hole dug by grave robbers in a space adjacent to the TT chamber (collected April 2004). All TT isolates and three KT ones were also accommodated in this clade, which includes soil dwellers (Domsch et al., l.c.). In light of these isolate data, members of the Harzianum-Virens clade may be involved in the biodeterioration of the TT murals, whereas members of both the the Harzianum-Virens and Viride clades may be involved in that of the KT murals.

Penicillium

Penicillium is one of the representative phialidic anamorph genera and is morphologically characterized by the penicillus (the brush-like conidiogenous apparatus) and chains of usually greenish ameroconidia (cf. Fig. 5). It is also associated with cleistothecial telemorphs (Talaromyces, Eupenicillium) of the Trichocomaceae, Eurotiales, Ascomycota. Members of Penicillium commonly inhabit the environment, including soil. Very recently, Domsch et al. (2007) enumerated 61 species as soil fungi.

Of ca. 200 Penicillium isolates from both tumuli, the predominant isolates were phenotypically identified as Penicillium sp. 1. Thirteen isolates of Penicillium sp. 1–7 from TT and 6 from KT–were selected for the molecular phylogenetic analysis. The D1/D2 LSU rRNA-sequence-based and ITS sequence-based phylogenies indicated that all TT and KT
isolates were assignable to Penicillium sect. Roqueforti (data not shown). Further, the β-tubulin sequence-based NJ phylogeny clearly demonstrated that all TT and KT isolates were grouped with the ex-type strain of \textit{P. paneum} Frisvad (Fig. 5). \textit{Penicillium paneum} has been isolated from moldy rye breads and other foods (Frisvad and Samson 2004). However, there are no reports on its interaction with the soil environment or on its biodeterioration of cultural properties (particularly murals). Therefore, this is the first report on the isolation \textit{P. paneum} from samples related to the biodeterioration of cultural properties. A paper on this subject will be published elsewhere.

Acremonium (sect. Gliomastix): the fungus causing the black stain on the Asuka beauties

The polyphyletic, microconidial genus \textit{Acremonium} is associated with over 10 teleomorphs of Hypocreales and others. It is characterized by the simple, erect phialides with hyaline or pigment-encrusted ameroconidia in slimy heads or dry chains (cf. Fig. 6). The taxonomy of \textit{Acremonium} is still controversial. Gams (1971) merged the anamorph genus \textit{Gliomastix} (Dickinson 1968) as a section into \textit{Acremonium}. We follow Gams’s (l.c.) concept of the genus \textit{Acremonium} in a very broad sense. \textit{Acremonium} sect. \textit{Gliomastix} is characterized by the presence of “chondroid hyphae” and usually darkly pigmented ameroconidia, and by a lack of chlamydospores. The Lascaux cave in France is faced with an invasion of black spots, from which \textit{Gliomastix} (= \textit{Acremonium} sect. \textit{Gliomastix}) and \textit{Ulocladium} were isolated (Dupont et al. 2007; Orial et al. in this book).

Micrographs (Fig. 6) show the cultural and morphological characteristics of the molds in question related to the cause of the black stains and spots on the murals (e.g., the Asuka beauties; see photo in figure). Based on these phenotypic characters, 32 TT and KT isolates producing chains of dark ameroconidia were assignable to \textit{Acremonium} (sect. \textit{Gliomastix}). The D1/D2 LSU rRNA gene-sequence-based phylogeny revealed the genealogical divergence of the TT and KT isolates. The TT isolates are very closely related to the KT isolates, but they differed morphologically and molecular-phylogenetically, suggesting they are of different species. We are still working very carefully to complete their accurate
identification at the species level. We call them Acremonium spp. herein.

**Comparison of mycobiota from inside and outside the TT chamber**

We classified the mycobiota samples collected after excavation of the mound and subsequent dismantling of the TT chamber after November 2006 into four categories, based on the sampling sites: the chamber interior (105 samples collected May 2004-December 2006), between the stone walls (47 samples collected May 2004-May 2007 and May 2004-July 2006, with the latter period including samples from the adjacent space), the chamber exterior (70 samples collected July 2006-May 2007), and the stone wall surface during and after the dismantling (23 samples collected Apr-May 2007). The results are summarized in Fig.7. There were some differences among the sample categories. *Penicillium* sp. I was isolated as the predominant growth in all the samples from inside and outside the chamber, whereas other species of *Penicillium* were isolated in large numbers from samples from between the walls and from outside the chamber. In addition, dematiaceous fungi, i.e., *Philalophora* and others, which were never isolated inside the chamber, were commonly found in samples from between the walls. In contrast, members of dark Acremonium, i.e., Acremonium (sect. Gliomastix), which had been related to the biodeterioration of murals and plaster walls, were scarcely isolated from samples from between walls or outside the chamber.

**Changes in bacteriobiota and their major colonizers of both tumuli**

Figure 8 shows the changes in bacteriobiota based on 50 samples of the TT chamber’s plaster walls collected from September 2005 to October 2006. For the period September 2005 to February 2006, the phyla *Fermicutes* and *Actinobacteria* were the most common bacteria. In that period, some environmental changes might have affected such changes in bacteriobiota as well as in fungi (as mentioned above). It is thought that one of the triggers of such changes was the cooling of the mound as a result of the dismantling of the chamber. *Ochrobactrum* (Teyssier et al. 2007) of the class *Alphaproteobacteria* was a dominant colonizer after September 2006,
Fig. 8 Changes in bacteriobiotas on the walls of the TT interior (September 2005-October 2006)

<table>
<thead>
<tr>
<th></th>
<th>05/09/16</th>
<th>06/02/20</th>
<th>06/05/17</th>
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<td>22</td>
<td>33</td>
<td>51</td>
<td>32</td>
</tr>
</tbody>
</table>

* The start of cooling of the burial mound

Fig. 9 Comparison of bacteriobiotas among the TT sampling sites

<table>
<thead>
<tr>
<th></th>
<th>Wall Surface before dismantlement</th>
<th>Space between walls</th>
<th>Outside of the stone chamber</th>
<th>Wall Surface after dismantling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples</td>
<td>50</td>
<td>18</td>
<td>17</td>
<td>12</td>
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<tr>
<td>Species</td>
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<td>21</td>
<td>27</td>
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</tr>
<tr>
<td>Isolates</td>
<td>152</td>
<td>53</td>
<td>41</td>
<td>26</td>
</tr>
</tbody>
</table>
whereas *Stenotrophomonas* (Palleroni and Bradbury 1993) of the *Gammaproteobacteria* after May 2006 was isolated as the major taxon. Species of both genera are widely distributed in nature. In addition, *Bordetella*, comprising mainly clinical species (and only one species, *B. petrii*, from the environment; von Wintzingerode et al. 2001) was isolated in July 2006. As shown in Fig.8, relative taxa of *Bacillus thuringiensis* (Claus and Berkeley 1986) of the class “Bacilli” (Firmicutes) were detected as the predominant colonizers throughout the period.

Figure 9 shows the differences in the bacteriobiota of inside- and outside-TT samples and their isolates from before to after the start of dismantling. In interior wall samples both before and after the dismantling work, *Proteobacteria* accounted for more than 50% of total isolates. On the other hand, in exterior and between-wall samples, *Firmicutes* and *Actinobacteria* accounted for 60%. *Bacillus aff. thuringiensis* was isolated from a large number of samples from the chamber’s interior and exterior, whereas *Bacillus aff. simplex* (Priest et al. 1988) was isolated only from samples that were not obtained from wall surfaces. In addition, *Stenotrophomonas* and *Ochrobactrum*, which were the major bacterial colonizers inside the interior, were also isolated from a large number of samples obtained from between the walls and the stone chamber exterior. Regarding samples of the adjacent space and room, *Ochrobacterum* (the phylum *Proteobacteria*), and *B. aff. thuringiensis* and the phylum *Actinobacteria*, were detected from the former and both, respectively. Relatives of the two major genera, i.e., *Stenotrophomonas* and *Ochrobactrum*, are associated with plants (the rhizosphere), and the clinical samples and the environment, respectively, whereas the remaining major genus *Bacillus* is composed of nonmedical (soil and others) and medical species, and insect pathogens (Dworkin et al. 2006a, b). Although the origins of the TT and KT isolates assignable to the three genera are still unclear, it is thought that these bacteria were brought into the stone chamber from the outside environment by some transporters, and colonized there.

![Fig.10 Morphological characteristics and molecular phylogenetic assignment of needle-like fruiting bodies on the stone wall of KT. Strain K4910-1: isolated from the stone wall surface of the KT adjacent small room (collected 10 September 2004). Bar = 0.02 substitutions/site. For other abbreviations, see Fig. 3.](image-url)
Morphological characteristics and molecular phylogenetic assignment of the selected fungi from the Kitora Tumulus

Identity of needle-like fruiting bodies on the stone wall: *Phialocephala* sp.

Two noteworthy fungi are mentioned below. One of these is a needle-like fungus that was observed on the walls of KT’s small room in September 2004 and grew there (Fig. 10). They were identified from morphological and molecular phylogenetic data of the needle-like fruiting bodies. Our integrated analysis based on the morphological characters and molecular phylogenetic data (D1/D2 LSU rRNA gene sequences) revealed that it is *Phialocephala phycomyces* (Auersw.) Kendrick (Kendrick 1964), which Jacobs et al. (2001) concluded is a distinct genus, *Kendrickiella* (Fig. 10). This fungus is strictly mitosporic or anamorphic and recorded from soil and plant samples (Kendrick i.e., Jacobs et al. i.e.). We have not yet arrived at a conclusion on the distinction between *Phialocephala* and *Kendrickiella*. Therefore, we temporarily take the former generic name in this paper. Fig. 10 also shows its vegetative hyphae that penetrated into the rock wall and that is difficult to remove because of the strong supporting structure. The same fungus was isolated from samples from between the walls and from the exterior of TT.

Identity of black particles on the ceiling: *Burgoa* sp.

The basidiomycete anamorph *Burgoa* produces compact, multicellular, thalloidic propagules called bulbils (Weresub and LeClair 1971). *Burgoa* is associated with the teleomorph genus *Sistotrema* of the Cantharellloid clade in the Basidiomycota (Hibbett and Thorn 2001, Moncalvo et al. 2006). Recently, new lichenicolous, muscicolous, corticolous, and lignicolous taxa of this genus were described by Diederich and Lawrey (2007).

The press initially described the mold’s appearance as “black spots” or “black particles”. It grew on parts of the star chart on the ceiling in April 2006. Afterward, it grew around and colonized on the walls of only the KT interior. Our integrated analysis based on phenotypic (morphological) and genotypic (D1/D2 LSU rRNA gene sequences) characters of
five selected isolates clearly revealed that they belong to a member of the genus *Burgoa* (Fig. 11). One of these micrographs clearly shows clamp connections in a hypha, which is characteristic of basidiomycetes. The isolates form small black bubils, which are characteristic of *Burgoa*. *Burgoa* spp. have been recorded from soil and plants (Weresub and LeClair 1971, Diederich and Lawrey 2007), hence they are thought to have invaded the chamber’s interior from the soil of the mound.

**Microbiota of biofilms and their major microorganisms**

Biofilms, which are usually thought of as slimy layers of microorganisms covering solid surfaces, are being studied from diverse aspects (e.g., de Beer and Stoodley 2006). Direct observations of both tumuli by the naked eye and with a loupe since September 2004 suggested that the plaster walls and mural paintings within the stone chamber were deteriorated by biofilms (initially called “viscous gel”; Fig. 12 in this paper and Fig. 8 on p. 49 in this book). Bacterial and fungal diversity and biodeterioration problems caused by biofilms colonizing walls and murals in Europe have been reported by Dornieden et al. (2000), Heyrman et al. (e.g. 2002, 2003a, b, 2005a, b), and Gorbushina et al. (2004). Heyrman (2003) and Heyrman et al. (l.c.) proposed several novel bacterial species from biofilms on murals in Spain, Austria, and Germany. Provided below are our systematic data obtained from an integrated analysis of phenotypic and genotypic characters of microbial isolates from biofilms inside both tumuli.

**Molds**

Figure 12 shows a biofilm appearing near the white tiger (*Byakko*) on the TT west wall (top) and a light-microscopic image of a sample (bottom). We isolated 35 biofilm samples from the TT chamber (collected July 2004-December 2006) and 32 samples from the KT chamber (September 2005 to September 2007) (cf. Table 3). We compared the mycobiota of the biofilms from both tumuli. As listed in Table 3, *Penicillium*, *Fusarium*, *Gliocladium* and yeasts were the major colonizers in the TT biofilms, whereas *Penicillium*, hyaline *Acremonium* (excl. sect. *Gliomastix*), and yeasts were predominant in the KT.

![Fig. 12 Photo (a) showing a biofilm (viscous gel; sample T5916-1, collected 16 September 2005) developed near the white tiger figure on the west wall of TT chamber and its micrograph (b) showing a mixture of hypha and microbial cells](image)
Fig. 13 Molecular phylogenetic assignment of yeast isolates from biofilms on the stone chamber interior walls of both tumuli. C. tumulicola T6517-9-5: isolated from biofilm on the lower part of the group on the east wall of the TT interior (collected 17 May 2006). C. takamatsuzukensis T4922-1-1: isolated as an airborne yeast of the TT interior (collected 22 September 2004). Bar = 0.05 substitutions/site.

Note: P. guilliermondii (19 strain) has been isolated from other samples of the TT stone chamber interior.

Fig. 14 Comparison of bacteriobiota from biofilms of the stone chamber Interior walls of both tumuli using the 16S rRNA gene sequence-based phylogeny. A, Ochrobactrum sp. T6220-2-3b with Gram staining; the strain was isolated from a black spot on the upper part of the Asuka beauties on the west wall of the TT interior (collected 20 February 2006). B, Stenotrophomonas sp. T5916-2-1b with the flagella stain; the strain was isolated from biofilm on the lower part of the hindfoot of the white tiger on the west wall of the TT interior (collected 16 September 2005). C, Bacillus sp. T5916-8-1b with Gram staining; the strain was isolated from brownish biofilm near the blue dragon on the east wall of the TT interior (collected 16 September 2005). Bar = 0.02 substitutions/site. *Isolated as the major taxa.
biofilms.

Yeasts

Figure 13 summarizes our results from isolation, phenotypic characterization, and D1/D2 LSU rRNA gene sequence analysis. The bootstrapped NJ phylogeny showed that yeast isolates from biofilms of the interior walls differed between TT and KT samples. Candida takamatsuzukensis, C. tumuliola, and Candida sp. (surrounded by a solid line in Fig.13) were isolated most frequently from the TT and KT, respectively. These Candida yeasts were suggested to be assignable to new species within the Candida membranifaciens clade (Suh et al. 2005). The two new species are characterized by positive assimilation of ethanol, nonassimilation of isopropyl alcohol, some strains isolated from biofilms, and the formation of a filamentous-yeast-like state. It is reported that many insect-associated yeast species were accommodated in the Candida membranifaciens clade (Suh et al. l.c.). It is possible that those three new species from TT and KT were brought inside both chambers by transporters, such as some insects (e. g. mites). Two species isolated from the Takamatsuzuka–C. tumuliola and C. takamatsuzukensis–were found to grow in repeated ethanol assimilation tests but not in isopropyl alcohol. As a result, treatment with isopropyl alcohol would more effectively disinfect the strains of C. tumulicola and C. takamatsuzukensis than ethanol. The well-developed septate true hyphae and pseudohyphae of C. tumulicola and C. takamatsuzukensis might play roles as biofilm substructures. Our proposal (Nagatsuka et al. 2009) on C. tumulicola and C. takamatsuzukensis spp. nov. has been appeared in the International Journal of Systematic and Evolutionary Microbiology.

Bacteria

We determined the phylogenetic positions of 50 and 32 bacterial isolates from biofilm samples from the TT and KT chambers, respectively, using the bootstrapped NJ analysis of 16S rRNA gene sequences. The NJ tree and cell morphology of Ochrobactrum sp., Stenotrophomonas sp., and Bacillus aff. thuringiensis are shown in Fig.14. Species diversity was found in both tumuli, and a clear difference was seen in the bacteriobiota between TT and KT biofilms. A very characteristic taxon, the class Betaproteobacteria, was isolated only from TT biofilms, not from KT ones. In comparison to TT biofilms, the class Alphaproteobacteria has shown more diversity in KT biofilms. As for Stenotrophomonas, a taxon close to S. maltophilia (Palleroni and Bradbury 1993) was found in the TT biofilms, while a taxon close to S. rhizophila (Wolf et al. 2002) was isolated from the KT ones. This means the genus was the same but the species differed. Bacillus (Claus and Berkeley 1986, Priest 1989), Rhizobium (Kuykendall et al 2005), and Streptomyces (Williams et al. 1989), which are common in the soil environment, were detected in both the TT and KT biofilms. These bacteria might somehow have invaded the chambers from the outside and colonized on the walls. The differences in environmental conditions between the chambers affected the biofilms’ bacterial species composition.

SEM observations on a plaster piece and biofilms on plaster

Two kinds of samples—a plaster piece and biofilms on the plaster—were used for our scanning electron microscopy (SEM) observations. The former sample, SEM-2, was of a plaster piece taken from between the west walls (collected no.: T7510-1, a plaster fragment in a space between the west 3 and the west 2 stone walls, collected May 10, 2007). The latter biofilm samples were as follows:

SEM-4-1: a black stain at the bottom right of a belt worn by the second woman from the right among the group of four Asuka beauties on the west wall, collected July 25, 2007, at the restoration facility.

SEM-4-2: a black stain near the right side of the belt worn by the Asuka beauty furthest to the right, collected ibid.

SEM-5: a black stain on the lower-right of the same woman, collected 7 August 2007 at the same facility.

The mounted samples were coated with a thin layer (ca. 5 nm) of platinum/palladium using an ion sputter coater (Hitachi E200). Specimens were observed in a Hitachi S-4000 SEM using an accelerating voltage of 5.0 kV at the Electron Microscopic Center, Tokyo University of Agriculture, Tokyo. The images were recorded using the most powerful digital image management software, Quartz PCI (Quartz Imaging, West Vancouver, BC, Canada)
Fig. 15  A plaster piece taken from a space between the west walls (sample T7510-1, collected 10 May 2007). SEM-2: Fungal hyphae and spores seen on a cross section and surface (film nos. 003661, 003659). The state of a plaster substratum and a mass of microbial cells (Film no. 003669).

Fig. 16  SEM-4-1: Dried biofilm (top), two small holes in which microbial spores are stuffed up (Film no. 003775), and the state of the bottom showing the growth of microbial cells (Film no. 003779). SEM-4-2: The state of the surface of dried biofilm (Film no. 003783), the state of the bottom showing the growth of numerous fungal hyphae (Film no. 003786) and the magnified phase (Film no. 003787). SEM-5: The surface of dried biofilm (top), the growth of microbial cells and spores (Film no. 003793), and the state of the cross section showing the invasion of fungal hyphae and microorganisms (Film no. 003800).
at various magnifications.

Figure 15, showing the fine structures of SEM-2, clearly demonstrates fungal hyphae and microbial cells penetrating the plaster; the microorganisms have damaged the texture of the plaster. On the other hand, Fig.16 shows fine structures of mainly dried biofilm surfaces on plaster pieces. For the respective states, see the legends of the SEM micrographs. As a whole, these SEM micrographs reveal the state of the biofilms: a mixture of molds, yeasts, and bacteria. The SEM observations confirm the isolation and identification data discussed above.

**Microbial community analysis by the DGGE method**

Finally, we examine the results of our microbial community analysis using the DGGE method, which is one of the uncultivation methods, to compare with those obtained from the cultivation methods (Muyzer et al. 1993, 1996). This molecular phylogenetic method has been applied to bacterial community analyses of murals in Europe (Gurtner et al. 2000, Schabereiter-Gurtner et al. 2002). DGGE has also been applied to characterize fungal communities of painted art objects by Möhlenhoff et al. (2001) and to analyze fungal communities in environmental and soil samples (van Hannen et al. 1998, Borneman et al. 2000, Hoshino and Matsumoto 2007). Our results for the TT samples using DGGE are provided below for bacteria and fungi.

**Bacteria**

The left side of Fig.17 shows the bacterial species composition inside and outside TT. The graph indicates the proportions at the phylum/class level among four sample categories (bamboo grove, exterior, between the stone walls, and interior), while this figure also indicates the major genera in the respective phyla/classes. From the interior, we recognized bacteria accommodated in the classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* and the phylum *Cyanobacteria*, but no bacteria were found of the class *Actinobacteria* or of the phyla *Firmicutes* and *Bacteroidetes*, which were common outside the

![Fig.17 Bacterial communities in TT samples obtained by the PCR-DGGE analyses. PCR-DGGE profiles of the right side show that two bacterial species were isolated from TT and also detected from DGGE bands (indicated by arrows).]
Fig. 18 | 16S rRNA partial gene sequence-based phylogenetic assignment of the Rhizobialean bacteria detected by the DGGE analyses and isolated by culturing. Bar = 0.01 substitutions/site. For details, see text.

Fig. 19 | Comparison of mycobiota inside and outside the TT chamber. Left: The fungal community analyses using nuclear SSU rRNA partial gene sequences and their DGGE band profiles. Right: Results from the BLAST search showing the proportions of fungal taxa detected.
chamber. The major bacterial taxa differed between inside and outside the chamber. Bacteria of the *Alphaproteobacteria* were recognized as the major colonizers in the chamber, and these are referred to as root nodule bacteria, such as *Ochrobactrum* (Lebuhn et al. 2000, Tripathi et al. 2006, Kämpfer et al. 2007).

The right hand of Fig.17 shows the DGGE images of the samples. The brown arrow shows a band of *Ochrobactrum* detected in samples from inside and outside the chamber. The green arrow shows a band of *Stenotrophomonas* detected in the east wall sample. These bacteria were also isolated by the pure culture method. Based on these results, *Ochrobactrum* bacteria were the major colonizers inside and outside the chamber, whereas *Stenotrophomonas* bacteria were dominant inside.

Figure 18 shows isolates of Rhizobial members (root nodule bacteria) and the bootstrapped NJ phylogeny inferred from 16S rRNA gene partial sequences derived from DGGE bands. A large number of isolates assignable to *Ochrobactrum* were isolated from the stone walls and other sample sites before the dismantling. The PCR-DGGE sequences derived from DNA extracted from the same samples of the stone walls were identical to those of *Ochrobactrum* and *Sinorhizobium*. In addition, they perfectly matched the isolates. In light of these results, we conclude that *Ochrobactrum* is one of the predominant bacterial genera of the samples in question. Using both the culturing and PCR-DGGE methods, the same bacterial species were detected in the samples before and after the dismantling. It is thought, therefore, that these bacteria in the soil environment invaded the interior.

**Fungi**

Figure 19 shows DGGE banding patterns using SSU rRNA gene partial sequences and fungal taxa detected from samples relating to the dismantling. The respective sequences of the bands with arrows were determined and a BLAST search was performed. The results are shown as a ladder chart on the right-hand side. Taxa of Ascomycota as the major and of Basidiomycota and Chytridiomycota as the minor were detected, but Zygomycota was not. Ascomycota accounted for 85% of the strains, and the orders Eurotiales, Hypocreales, and Chaetothyriales were evident in large portions. Eurotiales and Hypocreales were found in especially large ratios in TT, suggesting they are the major fungi (cf. the isolation and culture data mentioned above).

**Concluding remarks**

The predominant microorganisms relating to the deterioration of the stone chambers of both the Takamatsuzuka and Kitora tumuli were fungi (especially molds and yeasts) and bacteria. The predominant fungi of the TT samples for the period May 2005 to February 2006 were assigned to the phialidic anamorph genera *Fusarium*, *Trichoderma*, and *Penicillium*. *Fusarium* spp. and *Trichoderma* spp. decreased and were replaced by dark *Acremonium*, i.e., *Acremonium* sect. *Gliomastix*, after the cooling of the mound starting in September 2005. The top three bacterial genera, *Bacillus*, *Ochrobactrum*, and *Stenotrophomonas*, were detected for the same period. Similar changes in bacteriobiota occurred after the cooling of the mound. Viscous gels that developed on the stone walls of both the TT and KT interiors were biofilms comprising bacteria, yeasts, and molds; the major colonizers were identified. In addition, SEM observations on plaster and biofilm samples revealed fungal hypha and bacterial/microbial cells deeply penetrating the plaster texture. DGGE analysis, which is one of the uncultivation methods, was applied to elucidate the bacterial and fungal communities in some of the TT samples. The results from the cultivation and DGGE methods correlated well with each other. The latter method is useful for studying the species composition of microbiota inside and outside the chamber. In progress now are phenotypic and genotypic characterization and identification at the species level of the selected microbial isolates from the TT and KT samples. As was already demonstrated by Heyrman and Swings (2003) as well as in this article, all possible synthetic approaches in modern microbiology and related fields will help to elucidate the causes of the biodeterioration of the 1,300-year-old mural paintings and walls of the Takamatsuzuka and Kitora tumuli, while also explaining the mechanisms underlying microbial colonization and biofilm formation as well as the structures and composition of these substrates.
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