Stenotrophomonas tumulicola sp. nov., a major contaminant of the stone chamber interior in the Takamatsuzuka Tumulus

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During investigation of the biological contamination of the 1300-year-old mural paintings and plaster walls inside the stone chambers of the Takamatsuzuka and Kitora Tumuli (TT and KT) in Asuka-mura, Nara Prefecture, Japan, the identity of 17 bacterial isolates from blackish mouldy spots and viscous gels (biofilms) collected from both tumuli (16 isolates from TT and one from KT) during our 2005–2007 microbiological survey was systematically elucidated. One cluster of the major bacterial isolates was assigned to the genus Stenotrophomonas (class Gammaproteobacteria) by phylogenetic analysis of the 16S rRNA gene sequences. These isolates were divided into two groups A and B. Group A comprised 15 TT isolates that took a phylogenetic position near Stenotrophomonas chelatiphaga LPM-5T. Based on our analysis of the phenotypic (cultural, morphological, physiological and chemotaxonomic) characteristics and genotypic/molecular characteristics (DNA base composition, DNA–DNA relatedness, and 16S rRNA and gyrB gene sequences), the novel species name Stenotrophomonas tumulicola sp. nov. is proposed for the group A isolates with the type strain T5916-2-1bT (=JCM 30961T=NCIMB 15009T). Group B, which contained only one TT and one KT isolate, was closely related to [Pseudomonas] geniculata, [P.] hibiscicola, [P.] beteli, Stenotrophomonas maltophilia and Stenotrophomonas pavanii. The two isolates were genotypically and phenotypically assignable to S. maltophilia.

The Takamatsuzuka Tumulus (TT) and Kitora Tumulus (KT), which are thought to have been built sometime between the late 7th and early 8th centuries, are circular burial mounds located in Asuka-mura, Takaichi County, Nara Prefecture, Japan. TT and KT were excavated in 1972 and 2004, respectively, and designated Special Historic Sites by the government in 1973 and 2000, respectively. Both TT and KT are characterized by polychromal mural paintings on the plaster walls of their stone chamber interiors (TT: approx. 1.0 m wide, 2.7 m deep and 1.1 m tall; KT: approx. 1.0 m wide, 2.4 m deep and 1.2 m tall). Since the respective excavations, both stone chamber interiors have been exposed to microbial disturbances (e.g. moulds and bacteria) that eventually produced biofilms (or viscous gels) on the wall plaster of the chamber interiors (Kigawa et al., 2009, 2013, 2015; Sugiyama et al., 2009; Ishizaki & Kigawa, 2011). The stone walls of TT and KT have been relocated and the mural paintings are being repaired. In the course of our microbiological survey since May 2004, various bacteria, moulds and yeasts involved in the biodeterioration of the mural painting and plaster walls inside the stone chambers of both tumuli have been isolated from TT and KT samples using the same cultivation method (e.g. Sugiyama et al., 2009; Tazato et al., 2012; Nishijima et al., 2013). We obtained 365 bacterial isolates from 64 samples of TT and 42 samples of KT collected between September 2005 and April 2007 (Sugiyama et al., 2009) and September...
2005 and June 2006, respectively. The isolates were diverse and were assigned to six classes, namely the Actinobacteria, Bacilli, Bacteroidia, Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, and 48 isolates (32 from TT and 16 from KT) were assigned to the genus Stenotrophomonas (family Xanthomonadaceae, order Xanthomonadales, class Gammaproteobacteria) based on 16S rRNA gene sequence divergence, colony form and Gram staining. Stenotrophomonas isolates from TT were assigned to Stenotrophomonas sp. (initially indicated as 'sp. nov.' or 'sp. 1') as a major bacterial member and Stenotrophomonas malthophilia as a minor one, whereas those from KT were assigned to Stenotrophomonas rhizophila as a major bacterial member and S. malthophilia as a minor one, based on 16S rRNA gene sequence analysis (Sugiyama et al., 2009). In this study, we selected 17 Stenotrophomonas isolates related to Stenotrophomonas sp. and S. malthophilia (16 from TT and one from KT; cf. Table S1, available in the online Supplementary Material) as representatives for further study. We systematically elucidated the identity of the respective isolates at the species level based on their genotypic (molecular, i.e. 16S rRNA and gyrB gene sequences and DNA–DNA hybridization) and phenotypic (cultural, physiological and chemotaxonomic) characteristics. Based on our findings, we propose one novel species within the genus Stenotrophomonas, and assign two strains to one known species, S. malthophilia.

Each sample was suspended in sterilized physiological saline solution, spread on nutrient agar plates (CM3; Oxoid) and cultivated at 30 °C for 3 days under aerobic conditions. The 17 representatives of Stenotrophomonas isolates (Table S1) and reference strains of recognized Stenotrophomonas species (S. malthophilia NBRC 14161^T, Stenotrophomonas chelatiphaga DSM 21308^T, Stenotrophomonas koreensis JCM 13256^T, Stenotrophomonas nitritireducens JCM 13311^T and Stenotrophomonas acidaminiphila DSM 13117^T) were cultivated on nutrient agar or in nutrient broth (Oxoid) at 30 °C for 24 h for all tests, except for analysis of the cellular fatty acid (CFA) profile. Among the Stenotrophomonas isolates used in this study, the selected isolates, including the type strain of a novel species, have been deposited in two public culture collections (JCM in Tsukuba, Japan, and NCIMB in Aberdeen, UK), and the reference strains of known Stenotrophomonas species used for this study are listed (Table S1).

Almost-complete 16S rRNA gene sequences (1470 or 1472 bp) were determined according to previously described methods (Tazato et al., 2012) and these sequences were deposited in DDBJ (Table S1). The sequences were compared with those retrieved from DDBJ/ENA (EMBL)/GenBank by BLAST searches (Altschul et al., 1997). The sequences of the isolates and related species from GenBank were aligned using the MUSCLE program (Edgar, 2004). A phylogenetic tree was reconstructed by the neighbour-joining method with Kimura's two-parameter model using MEGA version 6.0 (Tamura et al., 2013). 16S rRNA gene sequences of the 17 representative isolates determined in this study were clearly revealed phylogenetically to be divided into two groups (A and B) in the Stenotrophomonas clade in the class Gammaproteobacteria (Fig. 1). The 16S rRNA gene sequence similarities of the isolates and closely related strains of known species are summarized in Table S2. Group A was composed of 15 isolates, namely T5916-2-1b^T, T6517-1-2b, T6517-2-2b, T6517-2-3b, T6713-4-2b, T6713-13-2b, T61017-1-2b, T61017-1-5b, T61017-2-2b, T61017-9-3b, T7214-12-2b, T7410-3-2b, T7417-2-1b and T7425-4-2b. Group B was composed of two isolates, K6303-5-2b and T6220-6-1b. The determined 1472 nt of group A were identical except for one nucleotide position among the 10 isolates including T5916-2-1b^T and five isolates (T6517-2-3b, T61017-1-2b, T61017-1-5b, T61017-2-2b and T7214-12-2b), whereas those of group B differed by only a single substitution. Levels of sequence similarity between groups A and B were 98.5–98.6 %; the two groups differed by two gaps, and 22 or 21 substitutions. These clear differences suggested that the 15 isolates of group A and the two isolates of group B are identical at the species level, respectively. The levels of 16S rRNA gene sequence similarity between group A and the type strains of closely related recognized Stenotrophomonas species were 96.9–99.2 %, with highest similarity to S. chelatiphaga LPM-5^T. On the other hand, the levels of gene sequence similarity between group B and recognized or misclassified species (Ramos et al., 2011) of Stenotrophomonas, namely [P.] geniculata, [P.] hibiscicola, [P.] beteli, S. malthophilia and S. pavani, were 98.8–99.7 %. These values suggest that group B is assignable to S. malthophilia, the type species of the genus. To clarify the clonality of the isolates of group A we determined and analysed the gyrB (DNA gyrase subunit B) gene sequence, which has previously been shown to be effective for species-level identification within the genus Stenotrophomonas (Svensson-Stadler et al., 2012). The phylogenetic trees based on the sequences of two regions (Regions 1 and 2) of the gyrB gene are shown in Figs S1 and S2, respectively. The representative sequences of the gyrB1 and gyrB2 genes of selected isolates of group A were determined according to the methods of Yamamoto et al. (2000) and Young et al. (2008), which were compared with those retrieved from DDBJ/ENA (EMBL)/GenBank by BLAST searches (Altschul et al., 1997). After multiple alignment using the MUSCLE program (Edgar, 2004), phylogenetic analyses of each gyrB1 and gyrB2 gene sequence, and the concatenated sequences (16S rRNA, gyrB1 and gyrB2 genes), were performed by the neighbour-joining method using MEGA version 6.0 (Tamura et al., 2013). The sequences of the gyrB1 gene (912 nt) and gyrB2 gene (861 nt) of the isolates were identical. Levels of gyrB1 gene sequence similarity between five TT isolates and Xanthomonas and Stenotrophomonas species were below 88 %, whereas the gyrB2 gene sequence similarity between five TT isolates and the most closely related S. chelatiphaga CCUG 57178^T (GenBank accession no. GU945531) was 84.1 %. Phylogenetic analysis based on the concatenated three gene sequences of the A isolates clearly showed these were accommodated within the genus Stenotrophomonas and took a position near S. chelatiphaga (Fig. S3).
The morphology of cells grown on nutrient agar at 30 °C for 24 h was observed by phase-contrast microscopy with a light microscope (BX50; Olympus). The Gram reaction, catalase and oxidase activities, and starch and Tween 80 hydrolysis were examined according to the methods of Cowan & Steel (1965). The presence, number and position of flagella were determined according to the method of Komagata (1985). The growth range at various temperatures (4–41 °C) and the pH range for growth (pH 4.0–10.0, using increments of 1.0 pH unit at 30 °C) were determined.

Substrate utilization as a sole carbon source and some physiological characteristics were determined by using the API 20NE and API ZYM galleries according to the instructions of the manufacturer (bioMérieux). Assimilation of L-histidine, cellobiose, lactose, trehalose, glutarate, pantothenate, β-hydroxybutyrate, L-aspartate, ethanol and isopropyl alcohol), and acid production from glucose or maltose were examined according to the method of Veron & Chatelain (1960).

Cells of isolates of groups A and B were Gram-negative, straight to curved rods, approximately 0.8 μm in width and 1.5–2.5 μm in length. Cells occurred singly or in pairs, were motile and had one or more polar flagella (Fig. S4). Spore formation of cells was not observed. Catalase activity was positive, and oxidase activity and nitrate reduction were negative. The cultural, physiological and biochemical characteristics of the two groups were compared with reference strains of species of the genus Stenotrophomonas (Table 1), and some differences from related species and from each other were recognized. Isolates of group A grew at 10–37 °C, but not at 4 or 41 °C, whereas those of group B and related species grew at 41 °C. Utilization of ethanol and isopropyl alcohol, and lipase (C4) activity of group A were different from those of group B and related species of the genus Stenotrophomonas.

The quinone system was determined by the HPLC method using a 996 PDA HPLC system (Waters) as described by Stenotrophomonas maltophilia K6303-5-2b (LC066104)
Stenotrophomonas maltophilia T6220-6-1b (LC066105)
[Pseudomonas] geniculata ATCC19397T (AB021404)
Stenotrophomonas africana CCUG 41684T (GU948534)
[Pseudomonas] hibiscicola ATCC 19867T (AB021405)
[Pseudomonas] beteli ATCC 19861T (AB021406)
Stenotrophomonas maltophilia NBRC 14161T (AB680569)
Stenotrophomonas pavanii ICB 89T (FJ478683)
Stenotrophomonas chelatiphaga LPM-5T (EU573216)
Stenotrophomonas tumulicola T5916-2-1b (LC066089)
T6517-1-2b (LC066090) and 13 other isolates
Stenotrophomonas ginsengisoli DCY01T (DQ109037)
Stenotrophomonas koreensis TR6-01T (AB166885)
Stenotrophomonas acidaminiphila AMX19T (AF273080)
Stenotrophomonas dacejoenensis MJ03T (GQ241320)
Stenotrophomonas humi R-32729T (AM403587)
[Pseudomonas] pictorum ATCC 23328T (AB021392)
Stenotrophomonas nitritireducens LgT (AJ012229)
Stenotrophomonas terrae R-32768T (AM403589)
Stenotrophomonas rhizophila e-p10T (AJ293463)
‘Stenotrophomonas panacium’ MK06 (GO856217)
Xanthomonas campestris LMG 568T (X95917)
Xylella fastidiosa ATCC 35879T (AF203391)
Pseudoanthomomas dokdonensis DS-16T (DQ178977)
Luteimonas nepitilis B1953/27.1T (AJ012228)
Arenimonas donghaensis HO3-R9T (DQ411038)
Dyella japonica XD53T (AB110498)
Frateuria aurantia IFO 3245T (AB091194)
Algiphilus aromaticivorans DG1253T (DQ486493)
Solimonas soli DCY12T (EF067861)

Fig. 1. Neighbour-joining tree inferred from 16S rRNA gene sequences (1360 nt), showing the positions of Stenotrophomonas tumulicola sp. nov. (isolates of group A), isolates of group B identified as S. maltophilia, other Stenotrophomonas species, and their related taxa. Bootstrap values above 50 % (expressed as percentages of 1000 replications) are shown on/below the respective branches. The type strains of Algiphilus aromaticivorans (family Algiphilaceae) and Solimonas soli (family Solimonadaceae) were used as outgroup taxa. Bar, 2 changes per 100 nt positions.

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Nishijima et al. (1997). The CFA profiles were determined by the standardized protocol of the MIDI system with the TSBA6 library (Sherlock Microbial Identification System produced by Microbial ID) by culturing on tryptone soy agar (Becton, Dickinson) at 30 °C for 24 h (Sasser, 1990). The predominant ubiquinone of isolates T5916-2-1b T and T61017-1-2b (group A), and isolates K6303-5-2b and T6220-6-1b (group B) was Q-8. The detection of Q-8 supports that these isolates are assignable to the genus Stenotrophomonas (Finkmann et al., 2000; Yang et al., 2006). The CFA profiles of selected isolates of group A and the phylogenetically closely related S. chelatiphaga DSM 21508^T are shown in Table S3. The CFA profiles of group A revealed that iso-C_{15:0} (28.4–30.2 %) was the major fatty acid, with C_{14:0} (12.0–12.3 %), summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 11.8–12.3 %), C_{16:0} (5.6–6.1 %) and anteiso-C_{15:0} (5.5–5.9 %) detected as the next most abundant components. The fatty acid profiles of group A isolates corresponded to those of Stenotrophomonas (Lee et al., 2011; Ramos et al., 2011) but were different from that of S. chelatiphaga in the ratio of iso-C_{15:0} and anteiso-C_{15:0} in particular. There were differences in the CFA profile of S. chelatiphaga determined by us and the original description (Kaparullina et al., 2009), which may be due to differences in the growth conditions and/or library used for the analysis.

The genomic DNA was extracted for DNA G+C content and DNA–DNA reassociation experiments according to the method of Marmur (1961). The extracted DNA was purified by equilibrium ultracentrifugation in CsCl/ethidium bromide gradients according to the method of Hamamoto & Nakase (1995) with modifications. The DNA G+C contents were determined according to the method of Tamaoka & Komagata (1984) using an SCL-10A vp HPLC system (Shimadzu). DNA–DNA reassociation experiments were performed using the photobiotin microplate hybridization method developed by Ezaki et al. (1989). The probe DNA was labelled with photobiotin and hybridization was performed at 51 °C in 2× SSC buffer containing 50 % (v/v) formamide on an immunoplate (Nunc). The fluorescence intensity was measured with a Microplate Reader photometer (GENios; Tecan) at a wavelength of 360 nm for excitation and 465 nm for emission. The resulting DNA G+C contents of isolates T5916-2-1b^T and T6517-1-2b belonging to group A, and of isolates K6303-5-2b and T6220-6-1b belonging to group B were 66.0 and 66.1 mol%, respectively (Table 1). The DNA G+C contents of the respective type strains of the closest relatives S. chelatiphaga and S. maltophilia were 68.3 mol% (Kaparullina et al., 2009) and 66.8 mol% (this study), respectively. The DNA G+C content of group A was lower than that of group B, i.e. S. maltophilia and S. chelatiphaga. DNA–DNA reassociations were examined with phylogenetic relatives among group A, group B, S. chelatiphaga DSM 21508^T and S. maltophilia NBRC 14161^T. Reciprocal DNA–DNA reassociation experiments were performed with the respective two novel isolates of groups A and B, S. chelatiphaga DSM 21508^T and S. maltophilia NBRC 14161^T. The results are shown in Table S4. DNA–DNA relatedness values between isolates T5916-2-1b^T and T6517-1-2b of group A were 82–83 %, whereas those between isolate T5916-2-1b^T and the phylogenetically closest relative S. chelatiphaga DSM 21058^T (Fig. 1) were 34–43 %. The levels of DNA–DNA relatedness between the two isolates of group A and S. maltophilia NBRC 14161^T ranged from 11 to 63 %, whereas those among two isolates K6303-5-2b and T6220-6-10b of group B and S. maltophilia NBRC 14161^T were greater than 71 %. In light of the 70 % recommended guideline for species delineation provided by Wayne et al. (1987), the two isolates T5916-2-1b^T and T6517-1-2b of group A are assignable to a novel species within the genus Stenotrophomonas, whereas the two isolates K6303-5-2b and T6220-6-10b of group B fall in the known species S. maltophilia.

On the basis of phenotypic characterization, 16S rRNA, gyrb1 and gyrb2 gene sequence-based analysis and DNA–DNA reassociation experiments, the isolates of group A represent a novel species of the genus Stenotrophomonas, for which the name Stenotrophomonas tumulicola sp. nov. is proposed. In addition, the phenotypic and genotypic (molecular) characteristics of the two Stenotrophomonas isolates of group B are identical to those of S. maltophilia (Palleroni & Bradbury, 1993): growth conditions, substrate utilization, enzyme production, acid production and other physiological characteristics (Table 1). The two strains of group B, K6303-5-2b (=JCM 30959) and T6220-6-1b (=JCM 30960), were isolated from a moulty spot on the KT stone chamber interior and a brownish viscous gel (biofilm) of the TT stone chamber interior, respectively (Table S1).

**Description of Stenotrophomonas tumulicola sp. nov.**

Stenotrophomonas tumulicola [tu.mu.li’co-la. L. masc. n. tumulus a little hill; L. suff. -cola (from L. n. incola) an inhabitant; N.L. n. tumulicola a tumulus dweller].

Cells are Gram-negative, strictly aerobic, motile, straight to curved rods, approximately 0.8 µm in width and 1.5–2.5 µm in length, and possess monotrichous polar flagella (Fig. S4). Colonies grown on nutrient agar (Oxoid) for 24 h are smooth, circular, non-glossy and yellowish and exhibit gliding motility. Growth occurs at pH 5.0–8.0 and at 10–37 °C, but not at 4 or 41 °C. Growth occurs in the presence of 5.0 % (w/v) NaCl. Cells cannot grow anaerobically. Catalase activity is positive, and oxidase activity and nitrate reduction are negative. Substrate utilization, enzyme production, acid production and other physiological characteristics (including differentiating characteristics from related species of the genus Stenotrophomonas) are shown in Table 1. Q-8 is the...
predominant ubiquinone and iso-C_{15}:0, C_{14}:0, summed feature 3 (comprising C_{16}:1\;\text{v}7\text{c} and/or iso-C_{15}:0\;\text{OH}) are the major cellular fatty acids. The G+C content of the genomic DNA is 66.0–66.1 mol\% (as determined by HPLC). All the isolates, including the type strain, identified as representing Stenotrophomonas tumulicola sp. nov., are listed in Table S1, together with data for related strains.

The type strain is T5916-2-1b\textsuperscript{T} (= JCM 30961\textsuperscript{T} = NCIMB 15009\textsuperscript{T}), isolated from a viscous gel (biofilm) appearing on the mural painting (Byakko) on the west wall inside the stone chamber of Takamatsuzuka Tumulus, Asuka-mura, Takaichi County, Nara Prefecture, Japan.

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**Table 1. Differential physiological characteristics between Stenotrophomonas tumulicola sp. nov. and phylogenetic neighbours in the genus Stenotrophomonas**

Strains: 1, S. tumulicola sp. nov. isolates (T5916-2-1b\textsuperscript{T}, T6517-1-2b, T6517-2-2b, T6713-4-4b, T61017-1-2b, T61017-9-3b); 2, S. maltophilia NBRC 14161\textsuperscript{T}; 3, S. maltophilia isolates (K6303-5-2b, T6220-6-1b); 4, S. chelatiphaga LPM-5\textsuperscript{T}; 5, S. pavani ICB89\textsuperscript{T}. Data for strains 4 and 5 were taken from Kaparullina et al. (2009) and Ramos et al. (2011), respectively. All strains were positive for catalase activity, hydrolysis of gelatin and utilization of sodium citrate, and were negative for growth at 4°C and urease activity. +, Positive; −, negative; w, weakly positive reaction; v, variable; NT, not tested; ND, no data available.

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<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>V (w)\textdagger</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>66.0, 66.1</td>
<td>66.8</td>
<td>66.3, 66.7</td>
<td>68.3</td>
<td>67.5</td>
</tr>
</tbody>
</table>

*The reaction occurs in only two strains, including the type strain T5916-2-1b\textsuperscript{T} and T6517-1-2b.
†The reaction in parentheses occurs in the type strain T5916-2-1b\textsuperscript{T}.

References


