Characterization of halotolerant and oligotrophic bacterial communities in Asian desert dust (KOSA) bioaerosol accumulated in layers of snow on Mount Tateyama, Central Japan

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Abstract: Microbial particles transported by Asian desert dust (KOSA) possibly impact ecosystems and human health in downwind environments and are commonly called "bioaerosols". The microbial communities associated with KOSA mineral particles (KOSA bioaerosol), which were collected from the snow cover on Mt. Tateyama, were investigated by means of a culture-amendment technique combined with denaturing gradient gel electrophoresis (DGGE) analysis using 16S rRNA genes. After the stratigraphy of the snow layer formed on the walls of a snow pit on Mt. Tateyama, samples were collected from 2 layers which included KOSA particles and one which did not. The snow samples with KOSA particles indicated microbial growth in the 100 and 10-1 dilution media and in the medium with NaCl below 10 %, while the snow sample without KOSA particles showed no microbial growth in the culture media. The PCR-DGGE analysis revealed that the bacterial compositions in the snow samples including KOSA mineral particles were mainly composed of the members of the phyla Actinobacteria, Firmicutus, and Proteobacteria. In particular, the 2 phylotypes appeared in the microbial cultures were similar to the members of the B. subtilis group, which has been detected in bioaerosol samples collected from the atmosphere over KOSA arrival (Suzu City) and source (Dunhuang City) regions. Presumably, halotolerant and oligotrophic bacterial communities are associated with the KOSA particles that descend to the snow cover on Mt. Tateyama.

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His research group monitored the abundance of cellular material and proteins in the atmosphere, demonstrating that the bioaerosol would occupy the total amount of aerosol.

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His research group demonstrated that bioaerosols at high altitudes may act as ice nuclei and cloud condensation nuclei affecting ice-cloud processes, and that the bioaerosols were transported to high attitude and were important factor for establishing the aerosol models.

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Response to Reviewers: Response for Reviewer

We thank you for your evaluation for publication of our manuscript. In the revised manuscript, we revised a part of text, and added the information of accession numbers of sequences.

[Q1] Please correct Akio and Watanabe in the text but Aoki and Watanabe in the reference list.

[A1] In the text of revised manuscript, "Akio and Watanabe" was improved to "Aoki and Watanabe" (P6L16).

[Additional improved sections]
The information of accession numbers of 16S rDNA sequences was inserted in the text (P9L8) and Table 2 in the revised manuscript.

Microbial particles transported by Asian desert dust (KOSA) possibly impact ecosystems and human health in downwind environments and are commonly called “bioaerosols”. The microbial communities associated with KOSA mineral particles (KOSA bioaerosol), which were collected from the snow cover on Mt. Tateyama, were investigated by means of a culture-amendment technique combined with denaturing gradient gel electrophoresis (DGGE) analysis using 16S rRNA genes. After the stratigraphy of the snow layer formed on the walls of a snow pit on Mt. Tateyama, samples were collected from 2 layers which included KOSA particles and one which did not. The snow samples with KOSA particles indicated microbial growth in the 10^{0} and 10^{-1} dilution media and in the medium with NaCl below 10 %, while the snow sample without KOSA particles showed no microbial growth in the culture media. The PCR-DGGE analysis revealed that the bacterial compositions in the snow samples including KOSA mineral particles were mainly composed of the members of the phyla *Actinobacteria*, *Firmicutus*, and *Proteobacteria*. In particular, the 2 phylotypes appeared in the microbial cultures were similar to the members of the *B. subtilis* group, which has been detected in bioaerosol samples collected from the atmosphere over KOSA arrival (Suzu City) and source (Dunhuang City) regions. Presumably, halotolerant and oligotrophic bacterial communities are associated with the KOSA particles that descend to the snow cover on Mt. Tateyama.

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2 Characterization of halotolerant and oligotrophic bacterial communities in Asian desert dust

3 (KOSA) bioaerosol accumulated in layers of snow on Mount Tateyama, Central Japan

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Abstract

Microbial particles transported by Asian desert dust (KOSA) possibly impact ecosystems and human health in downwind environments and are commonly called “bioaerosols”. The microbial communities associated with KOSA mineral particles (KOSA bioaerosol), which were collected from the snow cover on Mt. Tateyama, were investigated by means of a culture-amendment technique combined with denaturing gradient gel electrophoresis (DGGE) analysis using 16S rRNA genes. After the stratigraphy of the snow layer formed on the walls of a snow pit on Mt. Tateyama, samples were collected from 2 layers which included KOSA particles and one which did not. The snow samples with KOSA particles indicated microbial growth in the 10^0 and 10^{-1} dilution media and in the medium with NaCl below 10 %, while the snow sample without KOSA particles showed no microbial growth in the culture media. The PCR-DGGE analysis revealed that the bacterial compositions in the snow samples including KOSA mineral particles were mainly composed of the members of the phyla *Actinobacteria*, *Firmicutus*, and *Proteobacteria*. In particular, the 2 phylotypes appeared in the microbial cultures were similar to the members of the *B. subtilis* group, which has been detected in bioaerosol samples collected from the atmosphere over KOSA arrival (Suzu City) and source (Dunhuang City) regions. Presumably, halotolerant and oligotrophic bacterial communities are associated with the KOSA particles that descend to the snow cover on Mt. Tateyama.

Key words: KOSA, snow cover, bioaerosol, bacteria

1 Introduction

Asian desert dust (KOSA) occurred from the deserts of northern China, including the Gobi and Taklamakan Deserts, carry mineral-dust aerosol eastward over the East Sea to Japan and the Pacific Ocean by the west wind (Duce et al. 1980; Iwasaka et al. 1983). In addition to mineral dust, the microbial fractions associated with mineral-dust particles, which are commonly called "bioaerosols" include viruses, bacteria, fungi, and pollen as well as plant and animal debris (Jones and Harrison 2004; Jaenicke 2005). They potentially impact ecosystems and human health in the downwind environments (Prospero et al. 2005). Moreover, several species of microorganisms at high altitudes may act as ice nuclei affecting ice-cloud processes (Bowers et al. 2009; Pratt et al. 2009), which supports the possibility that wind-blown bioaerosol particles caught in dust events play an important role in atmospheric radiation transfer and the geochemical cycle of atmospheric constituents.

Recently, microbial cells attached to KOSA mineral particles have been observed with the use of an epifluorescence microscopic technique (Iwasaka et al. 2009) and the atmospheric-microbial compositions above the Taklamakan Desert (KOSA source region) were reported to be made up of several bacterial and fungal species (Kakikawa et al. 2009; Wang et al. 2010). Ground-based studies in KOSA arrival regions have also been undertaken to clarify the transport of microorganisms by KOSA events, demonstrating that the bioaerosol concentrations at downwind locations increased when the regions were impacted by airborne desert dust (Yeo et al. 2002). Our atmospheric studies conducted with balloons that collect samples at altitudes from 600 to 800 m recovered bacterial populations that were similar to those of the *Bacillus* sp. between the KOSA source (Dunhuang City) (Kobayashi et al. 2007; Maki et al. 2008) and

arrival (Suzu City) regions (Maki et al. 2010). Nevertheless, studies dealing with genetic diversity, KOSA season dynamics, global distribution, and ecological importance of airborne bacteria at KOSA arrival regions remain limited because the direct collection of KOSA particles from atmospheric areas requires a high level of sampling using balloons or aircraft.

During the winter and early spring, strong north-westerly winds result in heavy snowfall on Mt. Tateyama (3015 m above sea level), which faces the East Sea, and the snowfall sometimes includes KOSA particles from China. The depth of the snow cover on Mt. Tateyama is approximately 6–10 m in the spring (Aoki and Watanabe 2009). The air temperature, which rarely exceeds freezing from November to April, maintains the snow cover during the winter. Moreover, the snow cover prevents windblown contamination of local soil materials on the ground. Chemical analysis and meteorological correlations demonstrated that the deposition of KOSA mineral particles causes dusty dirt layers in the snow cover on Mt. Tateyama (Osada et al. 2004). Therefore, the snow cover is regarded as a product of continuous precipitation during the KOSA season, and sufficient numbers of atmospheric aerosol samples were obtained from the snow cover to analyze the microbial community associated with KOSA particles.

In the atmosphere, some microorganisms remain viable and are able to withstand low moisture, extreme temperature, limited oxygen, and extensive UV exposure (Imshenetsky et al. 1978; Alan and Harrison 2004). Halotolerant bacteria are known to tolerate high salinity and be resistant to stressors, such as high pH, extreme temperatures, and desiccation (Rothschild and Mancinelli 2001). The NaCl-amendment culture techniques recovered viable halotolerant bacteria at high altitudes in KOSA source and arrival regions (Maki et al. 2008, 2010). Halotolerant bacterial communities typify atmospheric microbial transport across hundreds to thousands of kilometers and at low to extreme altitudes (Okamoto et al. 2004; Echigo et al.

2005). On the other hand, since microbial communities under many different terrestrial and aquatic environments are often exposed to low-nutrient conditions, most environmental bacteria are assumed to be composed of oligotrophic bacteria, which survive through starvation-survival responses (Morita 1990). Oligotrophic bacteria produce a physical barrier to minimize environmental damage, such as extensive UV exposure (Blenkinsopp and Costerton 1991). An experimental design facilitating the cell activities of halotolerant and oligotrophic bacterial communities in bioaerosol samples is expected to be useful to investigate viable microorganisms transported by KOSA events.

In this study, snow samples including KOSA particles were collected from the snow cover on Mt. Tateyama. The viability of halotolerant and oligotrophic bacterial communities in the snow samples was evaluated with the amendment assay using culture media with different NaCl concentrations and different dilutions. The compositions of the bacterial species in the microbial cultures with culture-media amendment and in the genomic DNA of environmental snow samples were analyzed using denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified bacterial 16S rRNA genes (16S rDNA).

2. Materials and Methods

2.1 Sampling

The snow samples were collected at Murododaira (36.57N, 137.60E; 2450 m, MR) on Mt. Tateyama (Fig. 1). A snow pit was dug, and the walls of the pit were carefully smoothed to leave the stratigraphy of the snow layer undisturbed. After the surface snow on the wall was removed

using a sterilized snow sampler (polycarbonate plates: 3 cm x 20 cm x 0.1 cm), the 10 mL of snow sample was collected from 10 cm depth from the surface of snow wall using a new sterilized snow sampler. The snow samples were obtained from the 2 layers including mineral particles at 558 cm and 540 cm from ground surface (2 samples of Layers A and B, respectively), and from the single layer without sand particles at 249 cm (1 sample of Layer C) as a negative-control sample. On the snow wall, the snow layers that showed remarkable colours, e.g., brown-yellow or dark brown were selected as the layers including mineral particles. The sampling layers were composed of compacted snow, indicating that the samples had not melted and, therefore, maintained the records on deposition of aerosol from the atmosphere. The snow samples were allowed to melt at room temperature in the laboratory.

2.2 Environmental factors

The snow samples were allowed to melt in a laboratory, and their chemical compositions (anions and cations) were measured using ion chromatography (HIC-SP: SHIMADZU, Kyoto, Japan) (Aoki and Watanabe 2009). The values of nns-Ca were calculated from the concentration ratio of Na^+ to Ca^{2+}. The accuracy of the measured values was around 5%. The solutions of snow samples were also used for the following experiments.

The 500 μL solution of snow samples was fixed with a paraformaldehyde solution at a final concentration of 1 %. The samples were stained with DAPI (4',6-diamino-2-phenylindole) at a final concentration of 0.5 μg mL^{-1} for 15 min and filtrated through a 0.22 μm pore-size polycarbonate filter (Millipore, Tokyo, Japan) stained with Sudan Black (Russell et al. 1974). After the filter was placed on a slide on top of a drop of low-fluorescence immersion oil, a drop

of oil was added and then covered with a cover slide. Slides were examined using an epifluorescence microscope (Olympus, Tokyo, Japan) with UV excitation system. A filter transect was scanned, and the particles on the filter transect, which could be discriminated into water-insoluble particles in two size categories (mineral particles sized <5 μm and mineral particles sized >5 μm) and >5 μm water-insoluble particles with microbial-particle attachment (microbial aggregates sized >5 μm), were counted.

2.3 Physiological experiments

To investigate the viability of halotolerant bacteria, 1 mL of the solution of snow samples collected from the 3 snow layers was inoculated to 19 mL of Trypticase Soy Broth 10^0 ($TS10^0$) media and $TS10^{-1}$ media including NaCl at final concentrations of 0 %, 3 %, 10 %, and 15 % (w/v). The $TS10^{-1}$ medium was also prepared with a 10-time dilution of the $TS10^0$ medium. The tryptic-soy-broth (TS) medium was composed of 17 g trypticasepeptone, 5 g phytonepeptone, 2.5 g K_2PO_4, and 2.5 g glucose in 1 L of pure water. A TS medium has been used for detecting and isolating bacteria from bioaerosol samples (Maki et al. 2008). After the microorganisms in the bioaerosol samples were cultivated in the media at 20 ºC in the dark, the microbial growth was estimated using 550 nm absorbance every one or two days. After 13 days of incubation, 5 mL of the microbial cultures was used for determining species diversity by PCR-DGGE analysis targeting bacterial 16S rDNA.

2.4 PCR-DGGE analysis of bacterial 16S rDNA

Five mL of the solution of snow samples collected from 3 layers and 1 mL of the microbial cultures with culture-media amendment were used for the extraction of genomic DNAs using SDS, proteinase K, and lysozyme as described previously (Maki et al. 2008). Furthermore, the genomic DNAs were purified by phenol-chloroform extraction, chloroform extraction, and ethanol precipitation. A 16S rDNA region (ca. 550 bp) of the extracted genomic DNAs was amplified by PCR using the following oligonucleotide primers for PCR-DGGE analysis: GC-341F, 5'- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3'; and 907R, 5'-CCG TCA ATT CCT TT[A/G] AGT TT-3' (Muyzer et al. 1993). For each PCR reaction, 10 ng of the extracted DNAs was added to a PCR mastermix (20 μL) containing 2 μmol L^{-1} of dNTPs (Takara, Ohtsu, Japan), 2 nmol L^{-1} each of the primers, and 1U of Taq DNA polymerase (Takara, Ohtsu, Japan). Thermal cycling was performed using a Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan) with a thermal cycling program. Amplification was verified by agarose gel electrophoresis (1.5 % w v^{-1} agarose gel).

The DGGE analysis was performed with 6 % acrylamide gels containing a linear gradient of denaturant from 40 % to 60 % (100 % denaturant contained 7 mol L^{-1} of urea and 40 % (v/v) formamide). Electrophoresis was carried out at 60 °C and 90 V for 16 h in a 1 x TAE buffer with an electrophoresis system (AE-6290; ATTA, Tokyo, Japan). After electrophoresis, the gels were stained with SYBR Gold and scanned in a Printgraph (AE-6933FXCF: ATTA, Tokyo, Japan). Several bands on the gels were excised for sequencing. The excised gel pieces were transferred to PCR tubes, and the PCR amplicons (ca.550bp) were purified by phenol-chloroform extraction and chloroform extraction followed by ethanol precipitation. The nucleotide sequences were determined using a Dye Deoxy™ Terminator Cycle Sequencing Kit (ABI, CA,

USA) and a DNA autosequencing system (Model 373A, ABI, CA, USA) according to the manufacturer's recommended protocol. Primer 341F without a GC clamp or primer 907R was used as the sequencing primer. The determined sequences were compared with a DDBJ (DNA Data Bank of Japan) database using the BLAST and FASTA SEARCH programs. A phylogenetic tree including all sequences was constructed according to the neighbor-joining algorithmic method using TreeViewPPC (Saitou and Nei 1987). The DDBJ accession numbers for the partial 16S rRNA gene sequences from TTd-1 to TTd-15 are from AB575959 to AB575963, and those from TTd-16 to TTd-25 are from AB600636 to AB600643.

3 Results

3.1 Snow layers

The 3 layers on Mt. Tateyama where the samples were obtained were composed of compacted snow or solid-type depth boar from which no melted water was detected. Therefore, these snow layers maintained their original chemical and isotopic compositions at the time of the snowfall. The snow samples of Layers A and B included mineral particles sized <5.0 μm at particle densities of more than 4.00×10^6 particles m L^{-1}, while the mineral particles sized <5.0 μm of Layer C decreased to low particle densities of approximately 1.62×10^4 particles mL^{-1} (Table 1). Furthermore, the mineral particles sized >5.0 μm were also included in the snow samples of Layers A and B at particle densities ranging from 1.48×10^5 particles mL^{-1} to 3.76×10^5 particles mL^{-1}, and the particles sized >5.0 μm with microbial aggregates made up approximately 30 % of the total number of the particles sized >5.0 μm. In contrast, the large

mineral particles sized >5.0 μm were not observed in the snow samples of Layer C. These results indicate that the snow samples of Layers A and B included much higher quantities of mineral particles than those from Layer C (negative control).

The solutions of snow samples of Layers A and B contained Ca at significantly high concentrations of 164 μeq L^{-1} and 93.8 μeq L^{-1}, respectively, while the concentrations of Ca in Layer C were below the detection limit (Table 1). The 3 layers included Na^{+} at concentrations ranging from 5.42 μeq L^{-1} to 59.9 μeq L^{-1}. Most Na in the snow on Mt. Tateyama originates from sea salt. The contribution of sea salt Ca^{2+} to the total Ca^{2+} content was small (13 % on average). The solutions of snow samples of Layers A and B contained nns-Ca at high concentrations of 161 μeq L^{-1} and 57.6 μeq L^{-1}, respectively, while the concentrations of nns-Ca in Layer C were below the detection limit (Table 1). These results mean that the mineral particles in the snow layers specifically contained Ca which was tracer of KOSA mineral particles.

3.2 Physiological culture

The snow samples of Layers A and B including mineral particles showed microbial growth in the TS 10^{-1} media containing 0 % NaCl and the TS 10^{0} media containing 0 %, 3 % or 10 % NaCl within 13 days of incubation, while the snow sample of Layer C showed no microbial growth (Fig. 2). Moreover, none of the samples indicated growth in the media containing 15 % NaCl. In the TS 10^{-1} media with 0 % NaCl, the microbial growth of snow samples of Layers A and B increased remarkably to more than 30 absorbance (approximately 10^{8} cells mL^{-1}) within the 4 days of incubation, while microbial growth in the TS 10^{-1} media containing 3 %, 10 %,

and 15 % were not observed during the experimental period. In the TS 10^0 media containing 0 % and 3 % NaCl, the microbial growth of the snow samples of Layers A and B rapidly increased within 4 days of incubation and fluctuated between 64 and 190 absorbance (approximately 10^8 - 10^9 cells mL^{-1}) during the experimental period. The snow sample of Layer B in the TS10^0 media containing 10 % NaCl also showed microbial growth from the 6th day of incubation and maintained low values of absorbance of approximately 10 (approximately 10^7 cells mL^{-1}) during the experimental period.

3.3 DGGE analysis of the bacterial communities

PCR products amplified from the genomic DNA were directly extracted from the snow samples of Layers A and B, while PCR amplification was not obtained from the snow sample of Layer C, suggesting that the microbial biomass in the snow sample of Layer C was undetectable for PCR amplification. The DGGE analysis using the genomic DNA directly extracted from the snow samples revealed that the banding patterns were composed of more than 7 bands on each lane and that the bacterial population structure of Layers A and B was different (Fig. 3). On the other hand, the DGGE banding patterns of the microbial cultures with culture-medium amendment showed 1 to 3 bands on each lane. Most single bands (TTd-1, 4, 7, 10, 11, 12, and 14) occurred in the microbial culture and the genomic DNA directly extracted from snow samples of Layers A and B, suggesting that the same bacterial species were included in the microbial culture of the 2 snow layers. Two bands of TTd-8 and TTd-9 clearly appeared at each horizontal position in the microbial cultures with TS10^{-1} medium amendment of Layer A, and the bands at the same horizontal position were detected from the genomic DNA directly extracted from the snow

samples of Layers A and B (TTd-2, 3, 5, and 6). The 2 other bands of TTd-13 and TTd-15 were specifically detected from the 10 % NaCl-amended cultures of Layer A and the 3 % NaCl-amended cultures of Layer B respectively. These results indicate that some bacterial species in the microbial cultures amended with the $TS10^{-1}$ medium or 3 % and 10 % NaCl were different among the snow samples of the 2 layers.

We excised and sequenced the 25 bands from the DGGE gel. Thirteen phylotypes were obtained after comparing the sequences with each other and with the bacterial 16S rDNA databases, and composed of the members of the phyla *Fusobacteria*, *Actinobacteria*, *Firmicutus*, and *Proteobacteria* and the Plastid DNA (Table 2). Among all 13 phylotypes, 5 phylotypes were detected from the microbial cultures. A single phylotype of the 5 phylotypes was composed of the sequences of major bands which were detected from the genomic DNA directly extracted from the snow samples and the microbial culture of the 2 snow layers (TTd-1, 4, 7, 10, 11, 12, and 14), supporting the suggestion that the KOSA particles of the 2 snow layers contained the same bacterial species. Sequences of this phylotype belonged to the *B. subtilis* group and were identical to those of the *Bacillus* sp. SAd series that was detected at an altitude of 600 m in Suzu City during KOSA event (Table 2, Fig. 4). Moreover, the 2 other phylotypes, TTd-13 and TTd-15, which were specific to the microbial cultures with 10% NaCl and 3% NaCl respectively, also belonged to the genus *Bacillus* and were related with *B. megaterium* and *B. pumilus*, respectively. Among the remaining 2 phylotypes, one phylotype containing TTd-2, -5, and -8 was included in the beta-proteobacterial division relating to other known bacteria with a low similarity of below 98 %, and the other phylotype of TTd-3, -6, and -9 was identical to *Propionibacterium acnes* with a high similarity of 100 %. These 2 phylotypes were specifically detected from the microbial culture amended with $TS10^{-1}$ media (low-nutrient media),

suggesting the viability of oligotrophic bacteria in the snow samples.

The remaining 8 phylotypes (from TTd-16 to 25) of all 13 phylotypes were detected only from the genomic DNA directly extracted from the snow samples, and composed of several microbial species (Table 2, Fig. 4). The phylum *Fusobacteria* group and the plastid DNA included the sequences of TTd-16 and TTd-22 respectively, and were detected only from the genomic DNA directly extracted from the snow samples. Furthermore, in the phylum *Actinobacteria*, the two phylotyeps of TTd-20, 21, and 25 belonged to the genus *Corynebacterium* and the *Nocrdioides* group. The 3 phylotypes of TTd-17, 18, 19, 23, and 24 belonging to the beta-proteobacterial subdivision were related to the members of the genus *Neisseria*, the *Burkholderia* group, and the genus *Acidvorax*. These 8 phylotypes were not closely related to the other 5 phylotypes detected from the microbial cultures, suggesting that the some species of bacteria detected from the genomic DNA directly extracted from the snow samples disappeared in the microbial cultures.

4 Discussion

The KOSA mineral particles in the atmosphere were transported to Japan and accumulated in the snow cover on Mt. Tateyama from fall to spring (Osada et al. 2004; Aoki and Watanabe 2009). The snow samples of Layers A and B included a significant quantity of mineral particles sized >5 μm at higher particle densities of more than 15.0×10^4 particles mL^{-1} and showed high concentrations of nns-Ca (Table 1). In contrast, the mineral particles sized >5 μm and the nns-Ca in the snow samples of Layer C were below the detection limit. Highly alkaline Ca is a tracer of mineral dusts from desert and loess in China (Suzuki and Tsunogai 1993). In a previous

study, the chemical analysis of the composition of snow samples from Mt. Tateyama indicated the presence of preserved KOSA mineral particles (Osada et al. 2004). Presumably, the mineral particles of Layers A and B would be transported from Asian by KOSA events in the spring. The distribution of microorganisms in snow layers on Mt. Tateyama was of interest for identifying the bacterial species in the microbial communities associated with KOSA mineral particles.

The cell activities and growth of bacteria that survive in snow cover samples were facilitated using TS 10^0 and TS 10^{-1} media containing NaCl concentrations ranging from 0 % to 15 %. Among the samples in the 3 layers, those from Layers A and B included KOSA particles indicating microbial growth in the 0 %, 3 %, or 10 % NaCl media within 13 days of incubation, while the samples from Layer C included a few aerosol particles that showed no microbial growth in any medium (Fig. 2). The KOSA particles in Layers A and B are assumed to include bacterial cells that are tolerant to NaCl concentrations of up to 10 % and/or low-nutrient conditions. Bacterial populations which are tolerant to 10 % NaCl concentrations were also detected from bioaerosol samples collected at high altitudes in Dunhuang City (Maki et al. 2008) and Suzu City (Maki et al. 2010). Since most KOSA particles reaching Japan were mixed with salt over the East Sea and contained NaCl (Zhang et al. 2006), the microorganisms attached to KOSA particles in the atmosphere are expected to tolerate high NaCl concentrations. Echigo et al. (2005) reported that similar members of halotolerant bacteria inhabit non-saline environments in an area surrounding Tokyo and indicated that they may have been introduced by KOSA events. Moreover, bacteria that do not form spores or cysts survive under strict environmental conditions through a series of starvation-survival responses such as a reduction in cell size (Morita 1990). Starvation-survival appears to be a widespread response, as the bacteria that inhabit many different aquatic and terrestrial environments have been demonstrated

to survive the absence of growth nutrients (Morita 1990). Some oligotrophic bacteria are also reported to produce a physical barrier to separate adjacent areas of surrounding environments (Blenkinsopp and Costerton 1991). Such barriers might minimize environmental damage after exposure to UV irradiance in the atmosphere. Presumably, the halotolerant and oligotrophic bacteria were associated with KOSA particles and maintained their viability in the atmosphere and snow cover.

The PCR-DGGE analysis using 16S rDNA revealed that the microbial communities in the snow samples were composed of several bacterial species (Fig. 3). The sequences of bands on the DGGE gel were classified to the 13 phylotypes, of which 5 were detected from the microbial cultures (Table 2, Fig. 4). In particular, the 3 phylotypes were detected from the microbial cultures amended with 0 %, 3 % or 10% NaCl and belonged to the genus *Bacillus* in the phylum *Firmicutus* (Fig. 4). *Bacillus* sp. is known to form endospores, which are resistant to environmental stress and enhance their survival in the atmosphere (Riesenman and Nicholson 2000). They have been isolated from aerosol samples in various investigations worldwide as the most abundant genus (e.g., Prospero et al. 2005). In the *B. subtilis* group, a single phylotype detected from the major bands (TTd-1, 4, 7, 10, 11, 12, and 14) of both Layers A and B was closely related to the *Bacillus* sp. SAd series identified in aerosol collected from the atmosphere in the vicinity of Suzu City during KOSA event (Maki et al. 2010). Other phylotypes of TTd-13 and TTd-15 were similar to the *Bacillus* sp. DAd-1 and -11 in the *B. subtilis* group were dominant in the atmosphere in the vicinity of Dunhuang City (Maki et al. 2008). Some strains of *B. subtilis* isolated from the aerosol in Higashi-Hiroshima City (KOSA arrival region) and the Gobi Desert (KOSA source region) had a similar genetic identity, which supports the hypothesis of KOSA transport and deposition (Hua et al. 2007). *B. subtilis* have been recovered in the

aerosol collected from the northern Caribbean during African dust events (Kellogg et al. 2004). Moreover, the dominate phylotyepe of the *B. subtilis* group (TTd-1, 4, 7, 10, 11, 12, and 14) were detected by means of a culture-amendment technique combined with the PCR-DGGE analysis (Fig. 4, Table 2). In the previous investigations, when the atmospheric microbial communities at high altitudes in Suzu City were investigated by the culture-amendment technique, the viable bacterial species with NaCl amendment were similar to the bacteria detected from the DNA directly extracted from the bioaerosol samples and the most of bacterial communities belonged to the genus *Bacillus* (Maki et al. 2010). The findings of this study support the possibility that the bacterial population of the *B. subtilis* group can maintain its viability during atmospheric dust transport and extend its habitat toward Mt. Tateyama by KOSA events.

The members of the *B. subtilis* group sometimes cause nosocomial bacteremia by adsorption of oral preparations containing the bacterial spores (Richard et al. 1988) and are considered to be a major, primary and potentially serious pathogen infecting clinical patients (e.g., Velasco et al. 1992). In contrast, the *B. subtilis* group included the antagonists suppressing pathogenic diseases of plants (Alabouvette et al. 1996) or organic-matter degraders that contribute to the carbon cycle in terrestrial environments (e.g., Das and Mukherjee 2007). In addition, some strain types of *B. subtilis* have been used for the production of Japanese health foods such as *natto* (Ashiuchi and Misono 2002). In fact, the isolates of *B. subtilis* obtained from the snow samples in this study were demonstrated to produce *natto* (data not shown). Therefore, the atmospheric transport of the *B. subtilis* group might have negative and positive influences on humans and environmental ecosystems.

Among other 2 phylotypes that were specifically detected from the 2 bands (TTd-8 and

-9) of microbial cultures amended with $TS10^{-1}$ media (low-nutrient media) of Layer A, one phylotype was closely related with *Propionibacterium acnes* in the phylum *Actinobacteria*, and the other phylotype belonged to the beta-proteobacterial subdivision (Fig. 4). The phylotype of TTd-7 and -10 of the *B. subtilis* group was also detected from the microbial culture amended with the low-nutrient medium. These bacterial communities of the 3 phylotypes belong to oligotrophic bacteria, which survive through starvation-survival responses. In particular, the relative bacterial clusters of the phylotypes of TTd-2, -5, and -8 included *Ultramicrobacteria hongkongensis* and *Pandoraea* sp., which had a diameter of 0.3 μm and were hardly detected from the environmental samples (Silbaq 2009). Ultramicrobacteria are able to penetrate deeply into a porous matrix such as sandstone rock (Blenkinsopp and Costerton 1991). The atmospheric mineral particles might have originated from sandstone rock including ultramicrobacterial cells from the desert. Furthermore, relative bacterial species such as *Propionibacterium acnes*, in addition to *B. subtilis*, have been identified in aerosol collected from the northern Caribbean during African dust events (Griffin et al. 2003; Kellog et al. 2004). In particular, *P. acnes* is known to survive aerosol transport and is often detected in advanced noma lesions of humans, causing gangrenous disease in oral cavities (Paster et al. 2002). Moreover, *P. acnes* in microcomedo contribute to the development of the inflammatory phase of acne (Eady et al. 2003). Presumably, bacterial communities that did not form spores are associated with KOSA mineral particles and survive in the atmosphere and snow cover.

The remaining 8 phylotypes (from TTd-16 to 25) were detected only from the genomic DNA directly extracted from the snow samples. The microbial structures of 8 phylotypes were different from the microbial structures in the amended cultures of the snow samples. The spectrum of cultured isolates is narrower than that of diverse bacterial lineages detected using

culture-independent cloning and sequencing of 16S rDNA directly collected from aerosol samples (e.g. Maron et al. 2005). In general, 90% to 99% of bacteria in natural environments could not be cultivated by traditional methods and that many are viable but unculturable (Olsen et al. 1987). Among the 8 phylotypes, the 5 phylotypes were related to the opportunistic pathogens such as *Corynebacteirum* species (Feurer et al. 2004), *Neisseria* species (Smith et al. 1999), *Burholderia* species (Hayward et al. 1991) and *Leptorichia* species. (Collins et al. 2001). The relative bacteria of the 2 phylotypes of TTd-18, -19, and -24 were *Acidovorax* species (Willems et al. 1990) and *Burholderia* species (Castorena et al. 2006), of which some species were reported to degrade chemical compounds. The bioaerosols have the possibilities to implicate the human disease or the chemical-compound cycles in downwind ecosystems. The detection of plastid DNA of marine phytoplankton indicated that the seawater including phytoplankton would be mixed into the KOSA mineral particles in the atmospheric areas over the East Sea. The sequencing of 16S rDNA directly collected from snow samples would also provide the useful information about bioaerosols transported to Mt. Tateyama, although studies at the DNA level can not distinguished between viable microorganisms that cannot grow in culture media and nonviable microorganisms in the snow samples. The combination of the direct DNA extraction technique and the culture amendment technique is essential for clarifying the long-range transport of atmospheric bacteria by KOSA events.

This study demonstrates that KOSA particles in the snow cover of Mt. Tateyama are associated with viable halotolerant and oligotrophic bacteria. Halotolerant bacteria were found to be composed of members of the genus *Bacillus*, and the oligotrophic bacteria belonged to the genus *Bacillus*, the genus *Propionibacterium* and the beta-proteobacterial subdivision. The phylotypes of the *B. subtilis* group detected in this study were identical or similar to the

atmospheric bacteria collected at high altitudes in the regions of both the KOSA arrival (Suzu City) and source (Dunhuang City) (Maki et al. 2008, 2010). These results strongly support the possibility that the bacterial communities of the *B. subtilis* group are transported by the KOSA events. The sequencing of genetic DNA directly extracted from the snow samples suggested that the bioaerosol transported by KOSA events included unclturable microbial species, which were possibly related to opportunistic infection or chemical cycles in downwind ecosystems. The analytical data of the distribution of microorganisms in the snow layers on Mt. Tateyama have potential for use in bacterial transport research towards understanding the dynamics of atmospheric microbial communities during winter and spring, including KOSA events. However, the atmospheric data were insufficient to identify the accurate dates of KOSA events causing the accumulation of KOSA particles in the 2 snow layers. In the future, monitoring of the atmospheric aerosol dynamics in the vicinity of Mt. Tateyama from fall to spring will permit the identification of the origin of KOSA particles in each snow layer. Moreover, a comparison of the KOSA bioaerosols in snow cover with the microorganisms collected at high altitudes during the same KOSA event will clarify the long-range microbial transport from China to Japan by KOSA events.

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Figure Legends

Fig. 1 Map of Mt. Tateyama in Japan with a sampling site: Murododaira (2450 m, MR).

Fig. 2 Microbial growth of snow samples collected from Layers A (a, d), B (b, e) and C (c, f) in $TS10^{-1}$ (from a to c) and $TS10^{0}$ (from d to f) media containing NaCl at concentrations of 0 % (square), 3 % (circle), 10 % (triangle) and 15 % (diamond). All experiments were performed in tree bottles.

Fig. 3 DGGE profile (band patterns) of amplified 16S rDNA from the genomic DNA directly extracted from snow samples of Layers A and B, and from the bacterial cultures of Layers A and B amended with $TS10^{0}$ and $TS10^{-1}$ media including 0, 3, and 10 % NaCl. A 40 % (upper side) to 60 % (lower side) denaturing gradient was used.

Fig. 4 Phylogenetic tree including the partial sequences of 16S rDNA amplicons excised from the DGGE gel shown in Fig. 3. The tree was calculated from a dissimilarity matrix of ca. 342 bp (*Bacillus subtilis* numbering 455 to 786) alignment using a neighbor-joining algorithm. The accession number of each reference sequence is also given. Sample information (the microbial culture amended with $TS10^{0}$ and TS 10^{-1} media including 0, 3 and 10 % NaCl or the genomic DNA directly extracted from the bioaerosol sample) is shown in parentheses. Bootstrap values larger than 50 % (after 1,000 resampling) are indicated on the branches.

table

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Table 1. Concentrations of mineral particles and ions in the snow layers.

Layer	height	Concentrations of water insoluble particles (10^4 particles/mL)			Concentrations of ions (μeq L^{-1})		
		<5μm	>5μm	microbial aggregates (>5μm)	Na^+	Ca^{2+}	nss-Ca^{2+}
A	558	418 ± 11	21.0 ± 6.2	3.98 ± 1.88	59.9	164	161
B	540	444 ± 15	29.2 ± 8.4	5.57 ± 2.32	37.9	93.8	92.1
C	249	1.62 ± 0.57	N.D*	N.D*	5.42	0	0

*** Particles were not detected under microscopic observation.**

Table 2. Phylogenetic affiliation of sequences contained in the DGGE bands.

DGGE band No.*1	Sampling location*2	Sample conditon*3	Length (bp)	Category	GenBank accession no.	Closest relative	Similarity (%)*4
TTd-1, 4, 7 ,10, 11, 12, 14	A, B	$TS10^{-1}$ and $TS10^{0}$ media 0% and 3 %NaCl	432	*Firmicutes*	AB575959	*Bacillus* sp. SAd serise	100
TTd-2, 5, 8	A, B	$TS10^{-1}$ medium 0%NaCl direct extracted DNA	535	beta-proteobacteria	AB575960	*Ultramicrobacter hongkongensis*	97.3
TTd-3, 6, 9	A, B	$TS10^{-1}$ medium 0%NaCl direct extracted DNA	449	*Actinobacteria*	AB575961	*Propionibacterium acnes*	100
TTd-13	A	$TS10^{0}$ medium 10%NaCl	388	*Firmicutes*	AB575962	*B. pumilus*	100
TTd-15	B	$TS10^{0}$ medium 3%NaCl	414	*Firmicutes*	AB575963	*Bacillus* sp. B8W22	100
TTd-16	A	direct extracted DNA	464	*Fusobacteria*	AB600636	*Leptotrichia goodfellowii*	94.9
TTd-17	A	direct extracted DNA	473	beta-proteobacteria	AB600637	*Lautropia* sp. TeTO	99.9
TTd-18	A	direct extracted DNA	431	beta-proteobacteria	AB600638	*Burkholderia* sp. IMP5G	96.4
TTd-19, 24	A, B	direct extracted DNA	511	beta-proteobacteria	AB600639	*Acidovorax* sp. 01xTSA28A	97.2
TTd-20	A	direct extracted DNA	500	*Actinobacteria*	AB600640	*Corynebacterium tuberculostearicum*	99.4
TTd-21, 25	A, B	direct extracted DNA	519	*Actinobacteria*	AB600641	Actinobacterium OR-82	100.0
TTd-22	B	direct extracted DNA	460	Plastid DNA	AB600642	*Chrysochromulina polylepis* chloroplast gene	98.7
TTd-23	B	direct extracted DNA	483	beta-proteobacteria	AB600643	*Neisseria sicca*	99.0

*1 Numbers of the bands in Fig. 3 refer to the numbering of the TTd series.

*2 Layer of snow cover.

*3 Cultures cultivated with NaCl at concentrations of 0%, 3%, and 10%, and genomic DNA directly extracted from the bioaerosol samples.

*4 Similarity value between each isolate and the closest relative in databases.

line figure

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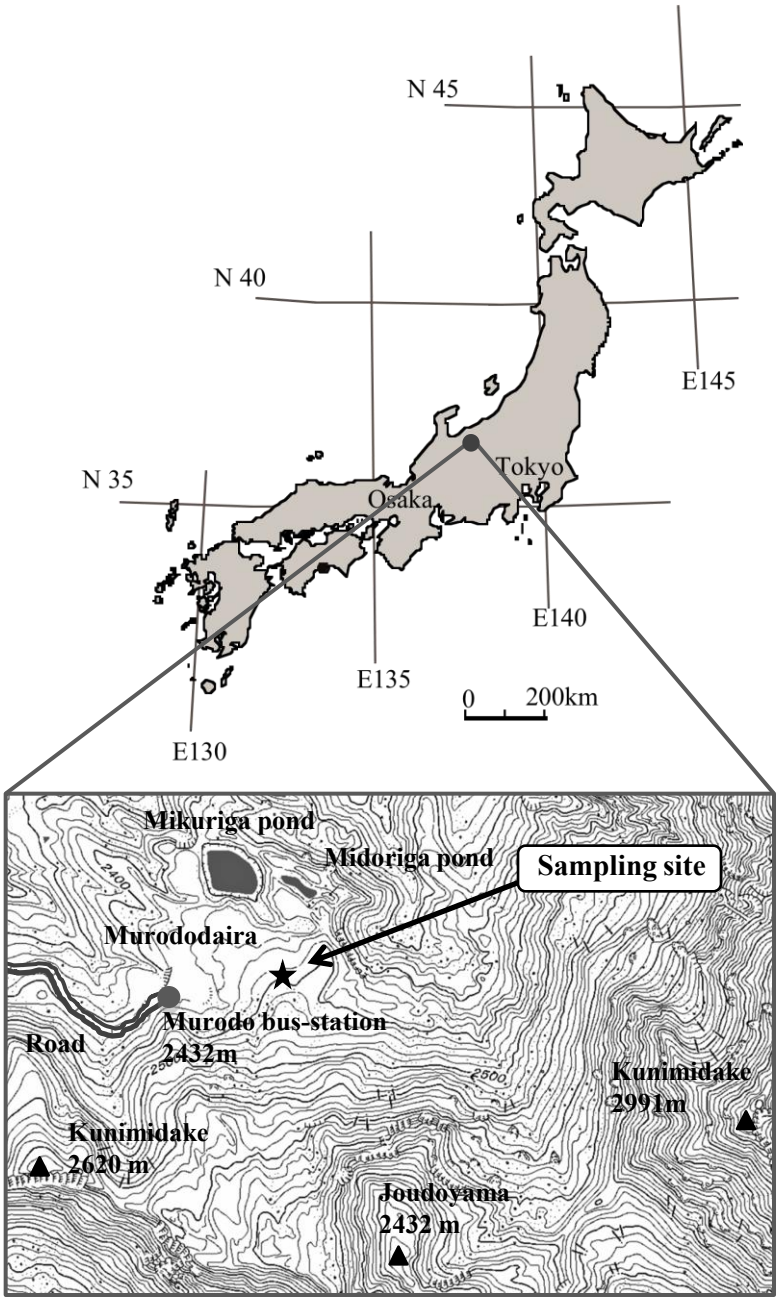

Fig. 1 T.Maki et al.

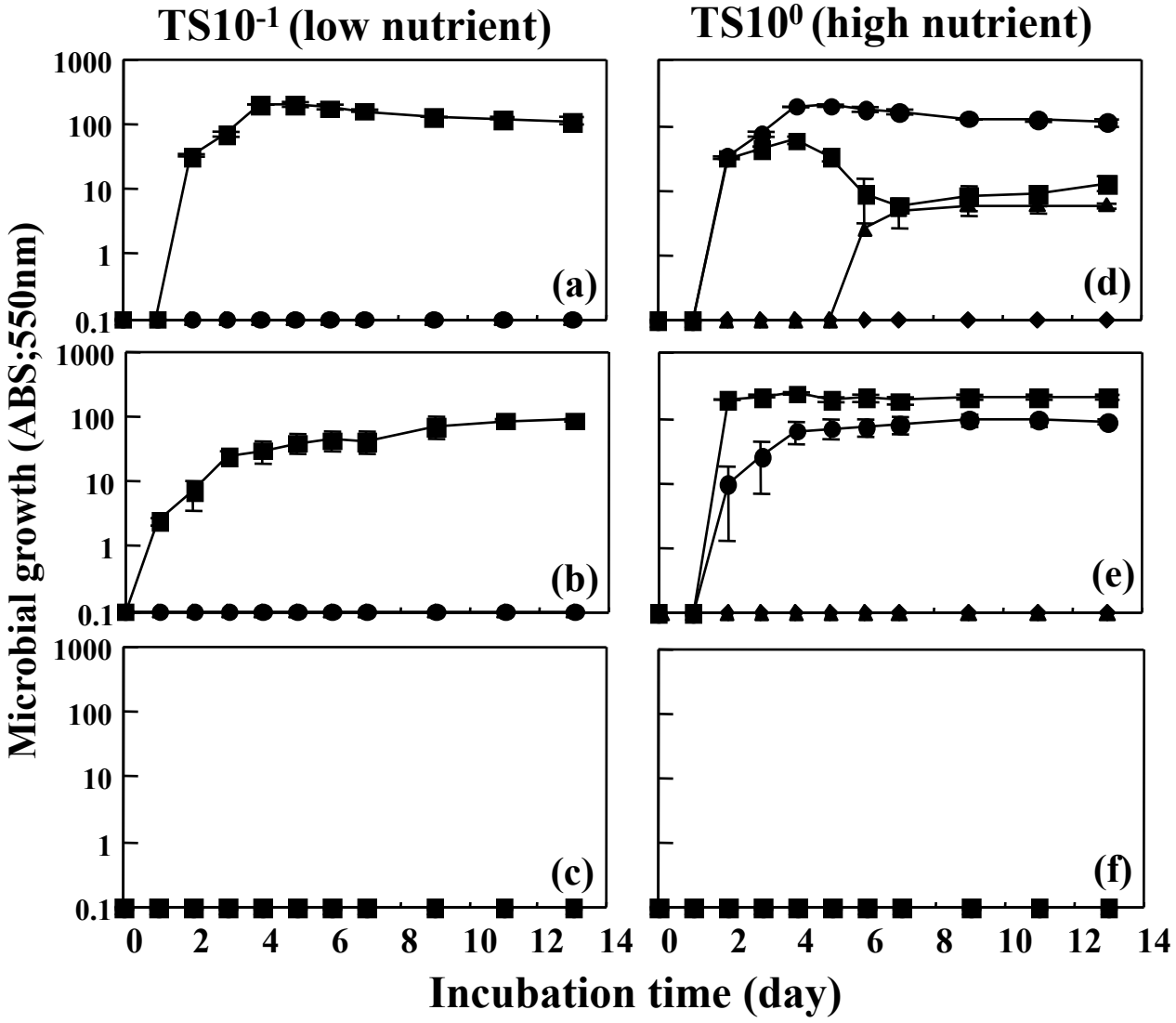

Fig. 2 T.Maki et al.

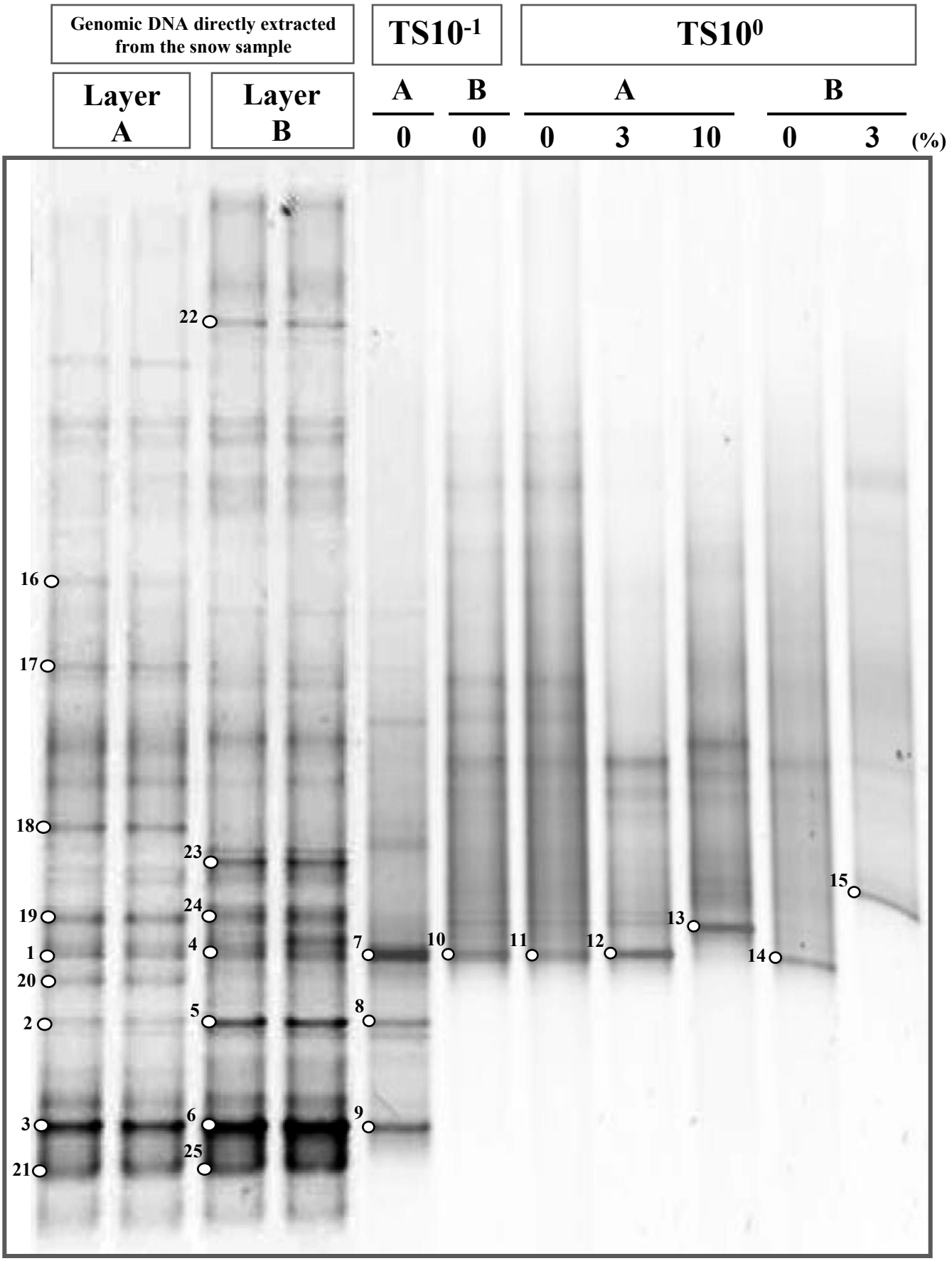

Fig. 3 T.Maki et al.

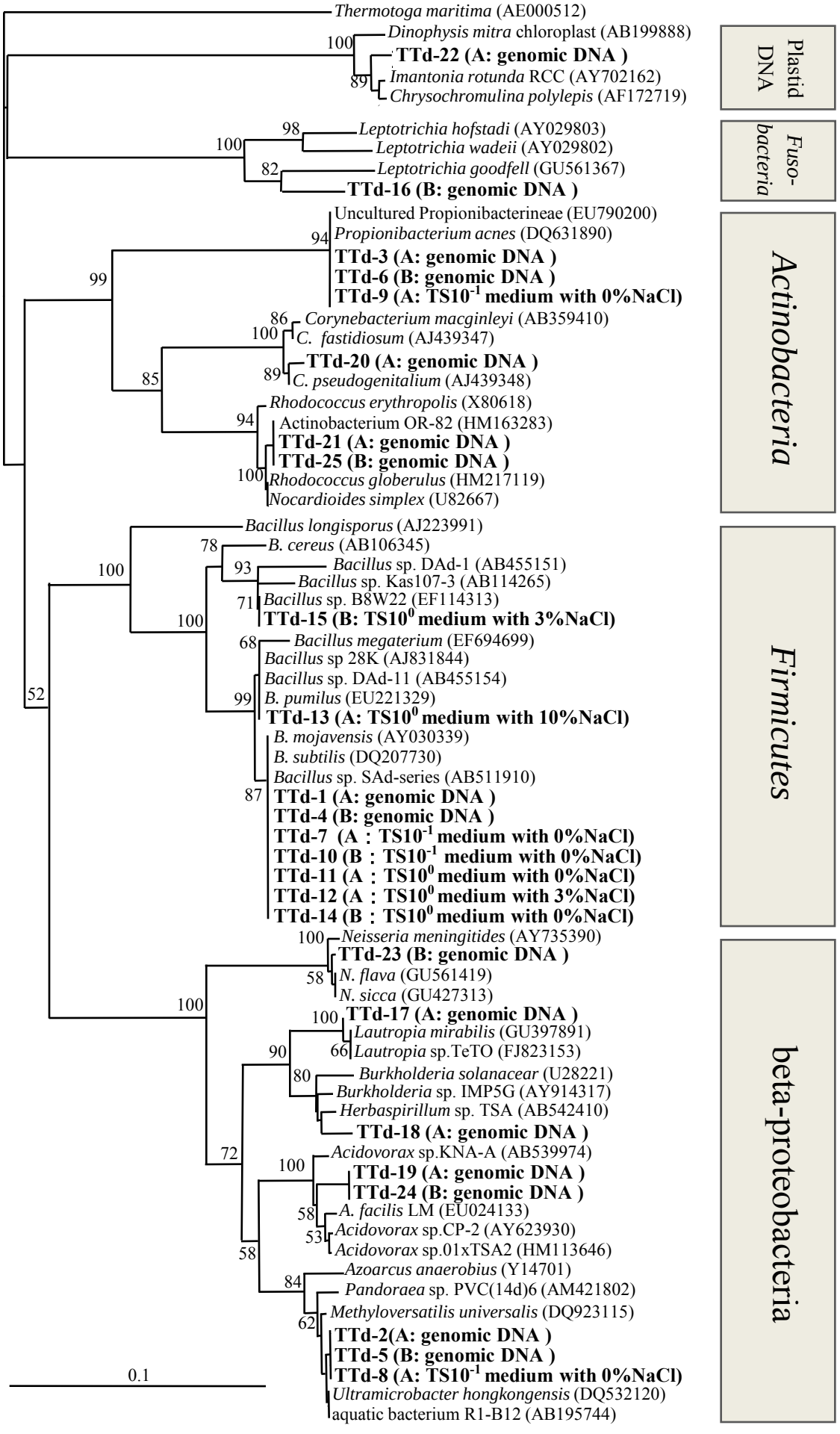

Fig. 3

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