

Phylogenetic analysis of atmospheric halotolerant bacterial communities at high altitude in an Asian dust (KOSA) arrival region, Suzu City

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Abstract: The microbial communities transported by Asian desert dust (KOSA) events have attracted much attention as bioaerosols because the transported microorganisms are thought to influence the downwind ecosystems in Korea and Japan. However, the atmospheric microbial community has not been investigated at high altitude in the KOSA arrival area. In this study, to estimate the viability and diversity of atmospheric halotolerant bacteria, which are expected to resist to various environmental stresses as well as high salinities, bioaerosol samples were collected at 10 and 600 m above the ground within the KOSA arrival area, Suzu City, Japan, during KOSA events. During the sampling period, the particle numbers at 600 m were higher than those at less than 20 m, suggesting that large particles of aerosol fall from the high altitude of 600 m to the ground surface. The microorganisms in bioaerosol samples grew in media containing up to 15

% NaCl concentrations demonstrating the viability of the halotolerant bacteria in bioaerosol samples. The PCR-DGGE analysis using 16S rDNA revealed that the bacterial species in NaCl-amended cultures were similar to the bacteria detected from the genomic DNA directly extracted from the bioaerosol samples. The 16S rDNA sequences of bacterial communities in bioaerosol samples were classified into 4 phylotypes belonging to the *B. cereus* or *B. subtilis* group. The bioaerosol samples collected at 600 m included 2 phylotypes belonging to *B. subtilis*, and one phylotype among all 4 phylotypes was identical between the samples at 10 and 600 m. In the atmosphere at 600 m, the halotolerant bacterial community would remain viable, and the species composition would include a few species of the genus *Bacillus*. During this investigation period, these atmospheric bacteria may have been vertically transported to the ground surface, where the long-range KOSA particle transport from China is frequently observed.

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Enclosed please find a manuscript (Phylogenetic analysis of atmospheric halotolerant bacterial communities at high attitude in an Asian dust (KOSA) arrival region, Suzu City), which we would like to submit to Science of the Total Environment. We would appreciate, if you ask reviewers to give consideration of its suitability for publication.

I will receive your correspondence, and please contact the following address.

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1 Title:

2 Phylogenetic analysis of atmospheric halotolerant bacterial communities at high attitude
3 in an Asian dust (KOSA) arrival region, Suzu City

4

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2

1 **Abstract**

2

3 The microbial communities transported by Asian desert dust (KOSA) events have
4 attracted much attention as bioaerosols because the transported microorganisms are
5 thought to influence the downwind ecosystems in Korea and Japan. However, the
6 atmospheric microbial community has not been investigated at high attitude in the
7 KOSA arrival area. In this study, to estimate the viability and diversity of atmospheric
8 halotolerant bacteria, which are expected to resist to various environmental stresses as
9 well as high salinities, bioaerosol samples were collected at 10 and 600 m above the
10 ground within the KOSA arrival area, Suzu City, Japan, during KOSA events. During
11 the sampling period, the particle numbers at 600 m were higher than those at less than
12 20 m, suggesting that large particles of aerosol fall from the high attitude of 600 m to
13 the ground surface. The microorganisms in bioaerosol samples grew in media
14 containing up to 15 % NaCl concentrations demonstrating the viability of the
15 halotolerant bacteria in bioaerosol samples. The PCR-DGGE analysis using 16S
16 rDNA revealed that the bacterial species in NaCl-amended cultures were similar to the
17 bacteria detected from the genomic DNA directly extracted from the bioaerosol samples.
18 The 16S rDNA sequences of bacterial communities in bioaerosol samples were
19 classified into 4 phylotypes belonging to the *B. cereus* or *B. subtilis* group. The
20 bioaerosol samples collected at 600 m included 2 phylotypes belonging to *B. subtilis*,
21 and one phylotype among all 4 phylotypes was identical between the samples at 10 and
22 600 m. In the atmosphere at 600 m, the halotolerant bacterial community would
23 remain viable, and the species composition would include a few species of the genus

1 *Bacillus*. During this investigation period, these atmospheric bacteria may have been
2 vertically transported to the ground surface, where the long-range KOSA particle
3 transport from China is frequently observed.

4

5 **Keywords** KOSA, Asian dust, bioaerosol, halotolerant bacteria, NaCl

6

7

1 **Introduction**

2

3 Asian desert dust (KOSA) that originates in desert regions of northern China, such
4 as the Gobi and the Taklamakan, can carry desert aerosols eastward over the East Sea to
5 Japan and the Pacific Ocean (Duce et al., 1980; Iwasaka et al., 1983) and possibly
6 impact ecosystems and human health in the downwind environments (Iwasaka et al.,
7 1988, Chung et al., 2003). In addition to mineral dust, the microbial fractions that are
8 transported by dust storms have received considerable attention in recent years
9 (Prospero et al., 2005). These fractions are commonly termed “bioaerosols” including
10 viruses, bacteria, fungi, and pollen as well as plant and animal debris (Jones et al., 2004;
11 Jaenicke 2005) and are reported to negatively influence plant and animal health and
12 cause diseases, such as asthma (due to allergens) or other illnesses (Griffin et al., 2004;
13 Kellogg et al., 2006; Griffin, 2007). Moreover, bioaerosols at high altitudes may act as
14 ice nuclei and cloud condensation nuclei affecting ice-cloud processes (Pratt et al.,
15 2009). Viable bacteria have been identified in super-cooled cloud droplets, and their
16 ability to grow in the environment has been confirmed (Sattler et al., 2001).

17 Bioaerosol research has developed very rapidly during the last two decades
18 (Mancinelli et al., 1987; Grinshpun et al., 2005), and data of microbial communities in
19 the atmosphere have primarily been accumulated in the Caribbean and Americas, which
20 are the most affected by dust storms originating in the Sahara and Sahel regions of
21 North Africa (Kellogg et al., 2004; Griffin et al., 2006). In addition, in recent times,
22 the atmospheric bioaerosols in the KOSA source area have also been investigated to
23 clarify the physiological characteristics (Iwasaka et al., 2009) and species composition

1 (Kakikawa et al. 2009) of microorganisms attached on KOSA particles. However,
2 only a few studies have focused on the atmospheric microbial communities in the
3 KOSA arrival regions, such as Korea (Yeo et al., 2002; Wu et al., 2004) and Japan, and
4 there is little information available on the microbial diversity in the atmosphere at high
5 altitudes in the KOSA arrival area.

6 In the atmosphere, some microorganisms remain viable and maintain their ability
7 to withstand low moisture levels, extreme temperatures, oxygen limitations, and
8 extended UV exposure (Imshenetsky et al., 1978; Alan et al., 2004). Halotolerant
9 bacteria are known to tolerate high salinity and be resistant to stresses, such as high pH,
10 extreme temperatures, or desiccation (Russell, 1989; Rothschild et al., 2001). In the
11 KOSA source area, Dunhang City, viable halotolerant bacteria have been detected using
12 NaCl-amendment culture techniques from bioaerosol samples collected at 600 m (Maki
13 et al., 2008). Halotolerant bacterial communities were targeted because they have
14 possibility to typify the atmospheric microbial transport across hundreds to thousands of
15 kilometers and at low to extreme altitudes (Okamoto et al., 2004; Echigo et al., 2005).
16 An experimental design facilitating the cell activities of a halotolerant bacterial
17 community in bioaerosol samples is expected to be useful for analyzing the
18 microorganisms transported by dust events.

19 In this study, bioaerosol samples were collected at heights of 10 and 600 m in Suzu
20 City during a KOSA event. The viabilities of halotolerant bacterial communities in the
21 bioaerosol samples were evaluated with the NaCl-amendment assay using culture media
22 at different NaCl concentrations. The compositions of the bacterial species in the
23 microbial cultures with NaCl amendment and in the genomic DNA of environmental

1 bioaerosol samples were analyzed using denaturing gradient gel electrophoresis
2 (DGGE) analysis of PCR-amplified bacterial 16S rRNA genes (16S rDNA).

3

4 **Experiment**

5

6 **Sampling**

7 Bioaerosol sampling was performed on the campus of the Suzu City (37.5°N,
8 137.4°E) on May 8 in 2008 (Fig. 1). Suzu City is located on the north coast of Noto
9 Peninsula in Japan, which is the arrival area of KOSA traveling from China. Aerosol
10 Index data were derived on the basis of the Ozone Monitoring Instrument (OMI)
11 (<http://jwocky.gsfc.nasa.gov/>) and backward trajectories calculated from the NOAA
12 Hybrid Single Particle Lagrangian Integrated Trajectory (HYPLIT) model,
13 (<http://www.arl.noaa.gov/HYSPLIT.php>). The bioaerosol sample collected at 600 m
14 above the ground was obtained using a tethered balloon (Kobayashi et al., 2007; Maki
15 et al., 2008). The bioaerosol sample collected at 10 m above ground was obtained
16 from the roof of a building. For collection at each altitude, we used an air pump with a
17 0.45 µm membrane filter and sampled 0.7 m³ of air for 1 hour during the daytime.
18 One hour of sampling time was the maximum time until the battery for the air pump
19 failed at 600 m in the atmosphere. Before the sampling, the filters were autoclaved
20 with a filter holder (In-Line Filter Holder, 47 mm; Millipore, Tokyo, Japan).
21 Environmental factors, such as the temperature and particle numbers in the atmosphere,
22 were determined using a thermometer (EX-501: EMPEX Instruments, Inc., Tokyo,
23 Japan) and a particle counter (KR-12A: RION CO., Ltd., Tokyo, Japan), respectively.

1 During the sampling period, the mean temperatures of the atmosphere at 600 m and 10
2 m were 15.7 ± 0.9 °C and 14.7 ± 0.9 °C, respectively, and the mean RH values were
3 58.3 ± 9.6 % and 80.6 ± 13.3 %, respectively. Within 1 hour of sampling, the bacterial
4 particles on the filter were washed off by shaking with 10 ml of sterilized water
5 containing 0.9 % (w/v) of NaCl. The washing solution was used for the determination
6 of the bacterial species composition by PCR-DGGE analysis. Moreover, the eluted
7 washing solution was utilized as a cultivation spike in media containing different NaCl
8 concentrations to investigate the viabilities of halotolerant bacteria.

9

10 **Physiological experiments**

11 To investigate the viabilities of halotolerant bacteria, 1 ml of the washing solution
12 was inoculated to 19 ml of TS media including NaCl at final concentrations of 0 %, 3
13 %, 10 %, and 15 % (w/v). The tryptic-soy-broth (TS) medium was composed of 17 g
14 trypticasepeptone, 5 g phytonepeptone, 2.5 g K_2PO_4 , and 2.5 g glucose in 1 liter of pure
15 water. A TS medium has often been used for detecting and isolating bacteria from
16 bioaerosol samples (Maki et al., 2008). After the microorganisms in the bioaerosol
17 samples were cultivated in the media at 20 °C in the dark, the microbial growth was
18 estimated using 550 nm absorbance every two days. After 15 days of incubation, 5 ml
19 of the microbial cultures was used for determining species diversity by PCR-DGGE
20 rDNA analysis.

21

22 **PCR-DGGE analysis of bacterial 16S rDNA**

1 One ml of the filter washing solution of bioaerosol samples collected at 10 and 600
2 m and 5 ml of the microbial cultures with NaCl amendment were used for the extraction
3 of genomic DNAs using SDS, proteinase K, and lysozyme as described previously
4 (Maki et al., 2008). Furthermore, the genomic DNAs were purified by
5 phenol-chloroform extraction, chloroform extraction, and ethanol precipitation. A 16S
6 rDNA region (ca. 550 bp) of the extracted genomic DNAs was amplified by PCR using
7 oligonucleotide primers for PCR-DGGE analysis, which were: F341-GC, 5'- CGC CCG
8 CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG
9 CAG CAG-3'; and R907, 5'-CCG TCA ATT CCT TT[A/G] AGT TT-3' (Muyzer et al.,
10 1993). For each PCR reaction, 10 ng of the extracted DNAs was added to a PCR
11 mastermix (20 µl) containing 2 µmol/l of dNTPs (TaKaRa, Ohtsu, Japan), 2 nmol/l each
12 of the primers, and 1U of Taq DNA polymerase (TaKaRa, Ohtsu, Japan). Thermal
13 cycling was performed using a Program Temp Control System PC-700 (ASTECC,
14 Fukuoka, Japan) with a thermal cycling program. Amplification was verified by
15 agarose gel electrophoresis (1.5 % w/v agarose gel).

16 The DGGE analysis was performed with 6 % acrylamide gels containing a linear
17 gradient of denaturant from 40 % to 60 % (100 % denaturant contained 7 mol/l of urea
18 and 40 % (v/v) formamide). Electrophoresis was carried out at 60 °C and 90 V for
19 16 h in a 1 x TAE buffer with an electrophoresis system (AE-6290; ATTA, Tokyo,
20 Japan). After electrophoresis, the gels were stained with SYBR Gold and scanned in a
21 Printgraph (AE-6933FXCF: ATTA, Tokyo, Japan). Several bands on the gels were
22 excised for sequencing. The excised gel pieces were transferred to PCR tubes, and the
23 PCR amplicons (ca.550bp) were purified by phenol-chloroform extraction and

1 chloroform extraction followed by ethanol precipitation. The nucleotide sequences
2 were determined using a Dye DeoxyTM Terminator Cycle Sequencing Kit (ABI, CA,
3 USA) and a DNA autosequencing system (Model 373A, ABI, CA, USA) according to
4 the manufacturer's recommended protocol. Primer F-341 without a GC clamp was
5 used as the sequencing primer. The determined sequences were compared with a
6 DDBJ (DNA Data Bank of Japan) database using the BLAST and FASTA SEARCH
7 programs. A phylogenetic tree including all sequences was constructed according to
8 the neighbor-joining algorithmic method using TreeViewPPC (Saitou et al., 1987).

9

10 **Nucleotide sequence accession numbers**

11 The DDBJ accession numbers for the partial 16S rRNA gene sequences are
12 AB511910 to AB511913.

13

14 **Results**

15

16 **Environmental factors**

17 The OMI aerosol index data averaged for 3 days (6-8 May 2008) indicated higher
18 values (>1.00) from KOSA source regions, such as the Gobi and Taklamakan Deserts
19 and the Loess Plateau, to KOSA arrival regions, such as Korea and Japan (Fig. 1). A
20 backward trajectory analysis for the sampling period was carried out using the NOAA
21 HYPLIT model. The backward trajectories revealed that the higher aerosol index
22 values originated from the desert or loess regions in China, in agreement with the OMI
23 image, suggesting that the prevailing westerly winds in the main troposphere brought

1 dust particles over KOSA arrival regions (data not shown). On the other hand,
2 according to the Japan Meteorological Agency, easterly surface winds of ~1 m/s were
3 recorded at less than 20 m above the ground in Suzu City during the sampling period.

4 The atmosphere at 600 m above the ground in Suzu City during the sampling
5 period included more aerosol particles with diameters exceeding 0.5 μm than were
6 observed at less than 20 m above the ground, while smaller aerosol particles with
7 diameters of 0.3 to 0.5 μm were fewer in number in the atmosphere at 600 m than in the
8 atmosphere at less than 20 m (Fig. 2). The size distributions of the particle numbers
9 were different in the atmosphere at 600 m and in that at less than 20 m above the ground,
10 indicating that atmospheric constituents at 600 m originated from areas other than Suzu
11 City. At heights of 600 m and less than 20 m, particles between 0.3 - 0.5 μm showed
12 high concentrations of more than 78,000 particles/l and made up about 90 % of the total
13 number of particles greater than 0.3 μm . In addition, the particles between 0.5 - 2.0
14 μm showed concentrations that were one order smaller than those between 0.3 - 0.5 μm
15 and ranged from 1,400 particles/l to 7,100 particles/l. Relatively large particles greater
16 than 2.0 μm were also detected at heights of 600 m and less than 20 m, and the total
17 concentrations ranged from 560 particles/l to 790 particles/l.

18

19 **Physiological culture**

20 When the bioaerosol samples were cultivated in a TS medium including different
21 NaCl concentrations, the microbial growth of both samples in the TS medium
22 containing 0 % and 3 % NaCl rapidly increased within 2 days of incubation and
23 fluctuated between 23 and 190 absorbances (approximately 10^8 - 10^9 cells/ml) during

1 the experimental period (Fig. 2). In the TS medium containing 10 % NaCl, the
2 microorganisms of both samples (10 and 600 m) gradually grew to over 80 absorbances
3 during 15 days of incubation. The cultures in the TS medium containing 15 % NaCl
4 started to show weak microbial growth from the 7th day and maintained low values of
5 absorbance of approximately 10 (approximately 10^7 cells/ml) during the experimental
6 period. These results indicated that the microorganisms that are tolerant to NaCl
7 concentrations of 3%, 10% and 15% maintained their viabilities in the aerosol samples
8 collected from 10 m and 600 m.

9

10 **DGGE analysis of the bacterial communities**

11 The bacterial species compositions in the bioaerosol samples were compared by
12 PCR-DGGE analysis targeting bacterial 16S rRNA genes (Fig. 3). The PCR products
13 amplified from the genomic DNA directly extracted from the bioaerosol samples
14 indicated different DGGE banding patterns in the bioaerosol samples collected from 10
15 and 600 m, and each banding pattern was composed of a single DGGE band (SDd-1 and
16 SDd-7, respectively) on the gel. These results indicate that different bacterial species
17 dominated in the samples collected at both altitudes. The bacterial communities of the
18 microbial cultures cultivated in the media containing 0 %, 3 %, 10 %, and 15 % NaCl
19 were also investigated using PCR-DGGE analysis (Fig. 3). The DGGE bands of
20 microbial cultures showed two dominant bands, and the banding patterns were also
21 different in the bioaerosol samples collected at 10 and 600 m, suggesting that the
22 bacterial community which grew in a saline media had different species compositions at
23 10 and 600 m. The microbial cultures of the bioaerosol samples collected at 600 m at

1 all NaCl concentrations tested commonly had the lower of the 2 bands (SDd-1, SAd-2,
2 SAd-3, SAd-5, and SAd-6), and this band was also detected from the bioaerosol
3 samples collected at 10 m at the 3 % NaCl concentrations tested. In contrast, the
4 microbial cultures of the bioaerosol samples collected at 10 m at all NaCl
5 concentrations tested showed the upper of the 2 bands (SDd-7, SAd-8, SAd-9, SAd-11,
6 and SAd-13), and the same located band also appeared in the bioaerosol samples
7 collected at 600 m at a 10 % NaCl concentration. The remaining band (SAd-12) was
8 specific to the microbial cultures of the samples collected at 10 m in the medium
9 containing 10 % NaCl. These results indicate that the dominant species composition
10 of the bacterial community in the atmosphere at 600 m is different from that in the
11 atmosphere at 10 m, while some species in the bacterial communities were common at
12 both altitudes.

13 We excised and sequenced 13 bands from the DGGE gel (Fig. 4). Four
14 phlotypes were obtained after comparing the sequences with each other and with the
15 bacterial 16S rDNA databases (Table 1). All sequences belonged to the genus of the
16 *Bacillus* bacterial group and clustered with the members of the *B. cereus* group and the
17 *B. subtilis* group (Fig. 4). A single phlotype among all 4 phlotypes included the
18 sequences of SDd-1, SAd-2, SAd-3, SAd-5, and SAd-6 from the bioaerosol sample
19 collected at 600 m and SAd-10 from the sample collected at 10 m, and their sequences
20 were identical to that of *Bacillus* sp. Z17 with a high similarity of 100 % and belonged
21 to the *B. subtilis* group. This result means that some species of bacterial communities
22 were common in the atmospheres at both altitudes. Another phlotype of 4 sequences
23 of SDd-7, SAd-8, SAd-9, SAd-11, and SAd-13 was specific to the bioaerosol samples

1 collected at 10 m and related to the bacterial members of the *B. cereus* group, such as *B.*
2 *cereus*, *B. thuringiensis*, and *B. mycooides*, with a high similarity of 100 %. Moreover,
3 SDD-7 was obtained from the DNA directly extracted from bioaerosol samples
4 suggesting that this phylotype would dominate in the atmosphere at 10 m. Another
5 sequence of SAd-4, which was obtained from the medium containing 10 % NaCl of the
6 bioaerosol sample collected at 600 m, indicated a low similarity of below 99.4 % to
7 other known bacteria in the DDBJ sequence database and was relatively related to the
8 members of the *B. subtilis* group. The remaining phylotype of SAd-12 obtained from
9 microbial cultures with 10 % NaCl of the samples collected at 10 m was closely related
10 to *B. subtilis* with a high similarity of 100 % and formed a cluster with the members of
11 *B. subtilis* group, including *B. amyloliquefaciens*, in the phylogenetic tree. These
12 results indicated that the bacterial community of the bioaerosol sample collected at 10 m
13 belonged to the *Bacillus cereus* group and the *B. subtilis* group, while only members of
14 the *B. subtilis* group were dominant in the samples collected at 600 m.

15

16 **Discussion**

17

18 Several results from field investigations (Griffin, 2007; Kellogg et al., 2006) have
19 suggested that microorganisms in the atmosphere were transported around the world
20 and influence ecological systems. The aerosol index data and the backward trajectory
21 supported the demonstration that some amounts of aerosol particles collected in Suzu
22 City were transported from KOSA source regions, such as the Gobi and Taklamakan
23 Deserts and the Loess Plateau, by prevailing westerly winds in the main troposphere

1 (Fig. 1). The relatively larger size of aerosol particles at heights of 600 m indicated
2 greater numbers there than at the heights of less than 20 m (Fig. 2), and the Japan
3 Meteorological Agency reported easterly surface winds of ~1 m/s at less than 20 m
4 above ground during the sampling period. Presumably, the aerosol particles in the
5 atmosphere at 600 m originated from other areas in the direction of the East Sea, while
6 those in the atmosphere at below 20 m mainly was transported from the east of Suzu
7 City. The vertical distribution of microorganisms in this KOSA arrival region (Suzu
8 City, Japan) during this sampling period was of interest for identifying the species
9 composition of the bacterial population transported from the Eastern Sea and China to
10 Japan.

11 Most previous studies on bioaerosols identified bacteria and fungi using traditional
12 culture methods, focusing on the fact that the number of culturable bacteria
13 in aerosol samples consistently increased during African dust events (Kellogg et al.,
14 2004; Griffin et al., 2006). However, it is recognized that 90% to 99% of bacteria in
15 natural environments cannot be cultivated by traditional methods and that many are
16 viable but unculturable (Olsen et al., 1987). The spectrum of cultured isolates is
17 narrower than that of diverse bacterial lineages detected using culture-independent
18 cloning and sequencing of 16S rDNA directly collected from aerosol samples (e.g.
19 Maron et al., 2005). A culture-independent method, which consisted of PCR-DGGE
20 analysis of bacterial genomic DNA directly extracted from environmental samples
21 (Muyzer et al., 1993), demonstrated that the bacterial species compositions were
22 influenced by the dynamics of environmental factors in atmospheric environments
23 (Maron et al., 2005). However, studies at the DNA level can detect both live

1 microorganisms that cannot grow in culture media and dead microorganisms that are
2 attached to dust particles. In this study, the experimental design carried out in the TS
3 media containing NaCl concentrations ranging from 0 % to 15 % facilitated the cell
4 activation and growth of halotolerant bacteria that survive in bioaerosol samples
5 collected at heights of 10 and 600 m. The PCR-DGGE analysis revealed that the
6 viable bacterial species with NaCl amendment were similar to the bacteria detected
7 from the DNA directly extracted from the bioaerosol samples and that the sequences of
8 this bacterial population were classified into 4 phlotypes belonging to the *B. cereus*
9 group and the *B. subtilis* group (Gram-positive bacteria) (Fig. 4, Table 1). The data
10 suggested that the atmospheric bacterial community over Suzu City is dominated by a
11 few Gram-positive bacterial species of the genus *Bacillus*. *Bacillus* sp. is known to
12 form endospores, which are resistant to environmental stress and enhance their survival
13 in the atmosphere (Riesenman et al., 2000). The desiccation conditions in the
14 atmosphere appear to require the spore-forming bacterial survival and reduce the
15 diversity of the microbial community.

16 Halotolerant bacteria, including Gram-positive bacteria are capable of survival in
17 extreme environments through resistance to several types of stress, such as desiccation,
18 UV irradiation, extreme temperatures, oxygen limitations and high salinity (Russell et
19 al., 1989; Rothschild et al., 2001). Furthermore, since most KOSA particles traveling
20 to Japan mixed with salt over the East Sea and contained NaCl (Tobo et al., 2009), the
21 microorganisms attached onto KOSA particles in the atmosphere are expected to
22 tolerate high NaCl concentrations. The PCR-DGGE analysis revealed that some of
23 the phlotypes of the genus *Bacillus* could grow in media with wide ranges of NaCl

1 concentrations. In particular, species of *Bacillus* are tolerant to wide ranges of NaCl
2 concentrations. Echigo et al. reported that similar members of halotolerant bacteria
3 inhabit non-saline environments in an area surrounding Tokyo, Japan and indicated that
4 they may have been introduced by KOSA events (Echigo et al., 2005). This
5 investigation suggested that, at high altitudes, extremely halotolerant bacteria would
6 maintain the viability and become to the strict environmental stresses of the atmosphere.
7 In a previous investigation at Dunhuang City in August 2007, Gram-positive bacterial
8 species of the genus *Bacillus* were isolated from bioaerosol samples collected at a
9 height of 800 m (Maki et al., 2008). Most of the genera cultured from the aerosol
10 samples in various worldwide investigations are Gram-positive bacteria (Shaffer et al.,
11 1997), including *Bacillus* spp. as the most abundant genus (e.g., Di Giorgio et al., 1996;
12 Prospero et al., 2005; Gorbushina et al., 2007). The bacterial populations detected
13 from the atmosphere in Suzu City may be transported from other regions, such as the
14 East Sea and China to spread their habitats around various environments.

15 The phylogenetic analysis using partial 16S rDNA sequences revealed that the
16 species compositions of major bacteria were different in the bioaerosol samples
17 collected at 10 and 600 m (Fig. 4). The sequences of the bioaerosol samples collected
18 at 600 m belonged to only the *B. subtilis* group, while those of the bioaerosol samples
19 collected at 10 m was composed of the phlotypes of the *B. subtilis* group and the *B.*
20 *cereus* group. However, a single phylotype that originated from dominant bacteria in
21 the bioaerosol samples collected at 600 m (the sequences of SDd-1, SAd-2, SAd-3,
22 SAd-5, and SAd-6) was also detected from the cultures with 3 % NaCl of bioaerosol
23 samples collected at 10 m (the sequence of SAd-10). Aerosol particles with a

1 relatively larger size observed at a height of 600 m would be transported from the west
2 of Suzu City, such as the East Sea and China (Fig. 2). Furthermore, the backward
3 trajectories and the Japan Meteorological Agency suggested different directions of wind
4 between 10 and 600 m (the easterly wind and the westerly wind, respectively) during
5 the sampling period. Therefore, bioaerosol particles in the atmosphere at 600 m are
6 thought to fall vertically to the ground surface. In a previous investigation, terrestrial
7 bacteria belonging to the genus *Bacillus* were isolated at 20,000 m above the ground,
8 supporting the idea of the vertical transport of microorganisms into the Earth's upper
9 atmosphere (Griffin, 2004). Therefore, mixing processes in the Japanese KOSA
10 arrival region may transport a part of the biomass of viable halotolerant bacterial cells
11 associated with mineral particles from the atmosphere to the ground in Suzu City.

12 The sequence of the phylotype detected from 10 and 600 m was identical to those
13 of *Bacillus* sp. Z17 and belonged to the bacterial members of the *B. subtilis* group.
14 The DDBJ sequence database indicated that *Bacillus* sp. Z17 was isolated from the
15 alpine grassland in China. Moreover, the phylotypes including SAd-4 and SAd-12 in
16 the cultures of bioaerosol samples collected at 10 and 600 m, respectively, were also
17 closely related to the members of *B. subtilis* group (Fig. 4). *B. subtilis*, which was
18 found in both the Higashi-Hiroshima City and the atmospheric dust source region of the
19 Gobi Desert, had a similar genetic identity, supporting the hypothesis of KOSA
20 transport and deposition (Hua et al., 2007). Relative bacterial species, such as *B.*
21 *subtilis*, have been identified in aerosol collected from the northern Caribbean during
22 African dust events (Kellogg et al., 2004) and from the Taklamakan Desert, which is a
23 KOSA source region (Kobayashi et al., 2007). The findings of this study support the

1 possibility that the relative bacterial population of *B. subtilis* can maintain its viability
2 during atmospheric dust transport and distribute its habitats toward Suzu City by
3 atmospheric dust transport.

4 The phylotype including SDd-7, SAd-8, SAd-9, SAd-11, and SAd-13 of the
5 bioaerosol sample collected at 10 m was identical to those of the bacterial members of
6 the *B. cereus* group (Fig. 4). Moreover, this phylotype, identified as *B. cereus*, was
7 also obtained from band SDd-7 of the DNA directly extracted from bioaerosol samples
8 collected at 10 m. This phylotype was not detected from the samples collected at 600
9 m and, accordingly, this phylotype would be specific to the dominant bacterial
10 community in the atmosphere at 10 m in Suzu City.

11 In this study, the viable halotolerant bacterial community in the atmosphere over
12 Suzu City during KOSA events was found to be composed of members of the genus
13 *Bacillus*. The relative members of *B. subtilis* group were demonstrated to dominate in
14 the atmosphere at 600 m in Suzu City and were detected from a single culture of
15 bioaerosol samples collected at 10 m. The members of the *B. cereus* group appear to
16 have their habitat in the atmosphere at 10 m, together with the members of the *B.*
17 *subtilis* group. Consequently, the falling processes through the boundary layer were
18 thought to transport viable cells associated with mineral particles from an altitude of
19 600 m to the ground surface in Suzu City during KOSA events. This bacterial
20 community may be carried by the wind over vast distances and travel with the KOSA
21 particles. The bacterial members of the genus *Bacillus* were detected in the
22 atmosphere of both the KOSA arrival region (Suzu City) and the KOSA source region
23 (Dunhuang City), but the bacterial 16S rDNA sequences detected in this study were not

1 perfectly identical to the bacterial sequences obtained from the bioaerosols of Dunhuang
2 City in the course of our investigation in August 2007 (Maki et al., 2008). This
3 inconsistency of bacterial species is thought to be the result of differences in the
4 sampling periods. In the future, during the same KOSA event, bioaerosols at high
5 altitudes in both the KOSA arrival and the KOSA source regions will be analyzed to
6 demonstrate the long-range microbial transport from China to Japan by KOSA events
7 and explain the ecological changes and possible affects on human and animal health in
8 downwind environments.

9

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11

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17

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- 13
- 14

1 Figure Legends

2

3 Fig. 1 OMI aerosol index data averaged for 3 days (6-8 May 2008).

4

5 Fig. 2 Particle concentrations of each diameter size in the atmosphere at 600 m (white
6 bars) and to less than 20 m (grey bars) above the ground.

7

8 Fig. 3 Microbial growth of bioaerosol samples collected at heights of 600 m (a) and
9 10 m (b) in media containing NaCl at concentrations of 0 % (square), 3 % (circle), 10 %
10 (triangle) and 15 % (diamond). All experiments were performed in five test tubes.

11

12 Fig. 4 DGGE profile (band patterns) of amplified 16S rDNA from genomic DNA
13 directly extracted from bioaerosol samples at 10 and 600 m and from the bacterial
14 cultures of bioaerosol samples collected at 10 and 600 m, which were cultivated in TS
15 media containing 0 %, 3 %, 10 %, and 15 % NaCl. A 40 % (upper side) to 60 %
16 (lower side) denaturing gradient was used.

17

18 Fig. 5 Phylogenetic tree including the partial sequences of 16S rDNA amplicons
19 excised from the DGGE gel shown in Fig. 4. The tree was calculated from a
20 dissimilarity matrix of ca. 409 bp (*Escherichia coli* numbering 421 to 848) alignment
21 using a neighbor-joining algorithm. The accession number of each reference sequence
22 is also given. Sample information (NaCl concentrations in the culture medium or
23 genomic DNA directly extracted from the bioaerosol sample) is shown in parentheses.

- 1 Bootstrap values larger than 50 % (after 1,000 resampling) are indicated on the
- 2 branches.
- 3

Table 1. 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}
SDd-1 SAd-2, 3, 5, 6, 10	10m and 600m	<15%NaCl direct extracted DNA	553	Gram- positive group	AB511910	<i>Bacillus</i> sp. Z clade	100
SAd-4	600m	10%NaCl	409	Gram- positive group	AB511911	<i>B. mojavensis</i>	99.4
SDd-7 SAd-8, 9, 11, 13	10m	<15%NaCl direct extracted DNA	544	Gram- positive group	AB511912	<i>B. cereus</i>	100
SAd-12	10m	10%NaCl	563	Gram- positive group	AB511913	<i>B. subtilis</i>	100

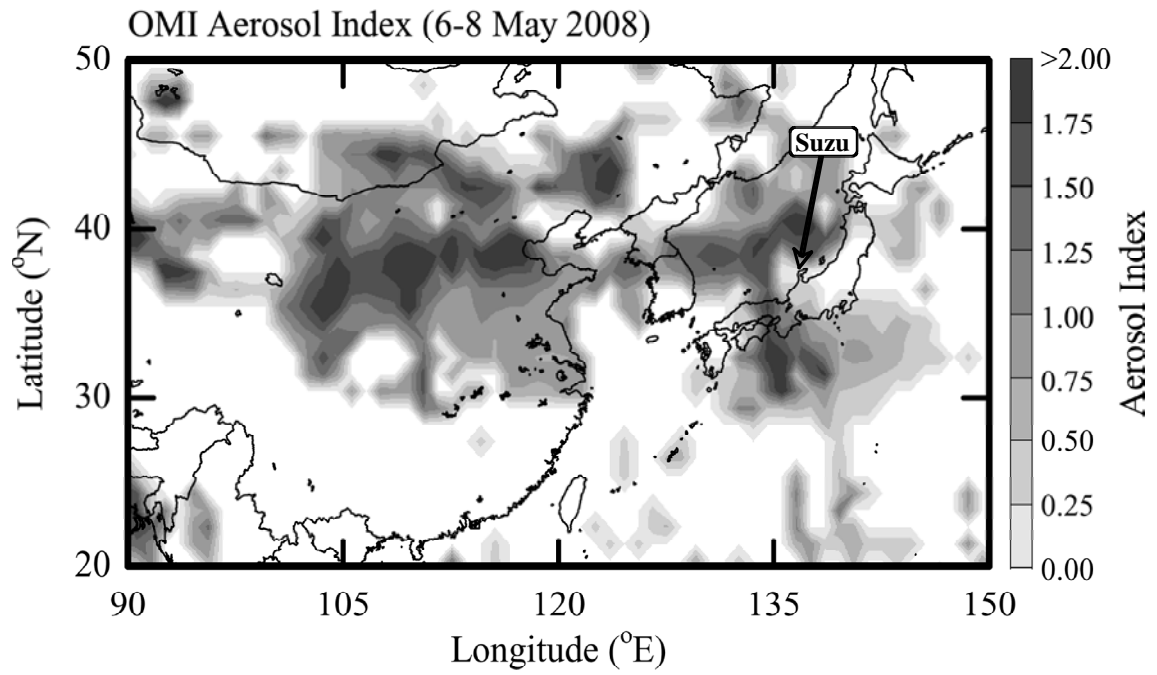
*1 Numbers of the bands in Fig. 4 refer to the numbering of the SAd or SDd series.

*2 Height above ground.

*3 Cultures cultivated with NaCl at concentrations of 0%, 3%, 10% and 15%, and genomic DNA directly extracted from the bioaerosol samples.

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

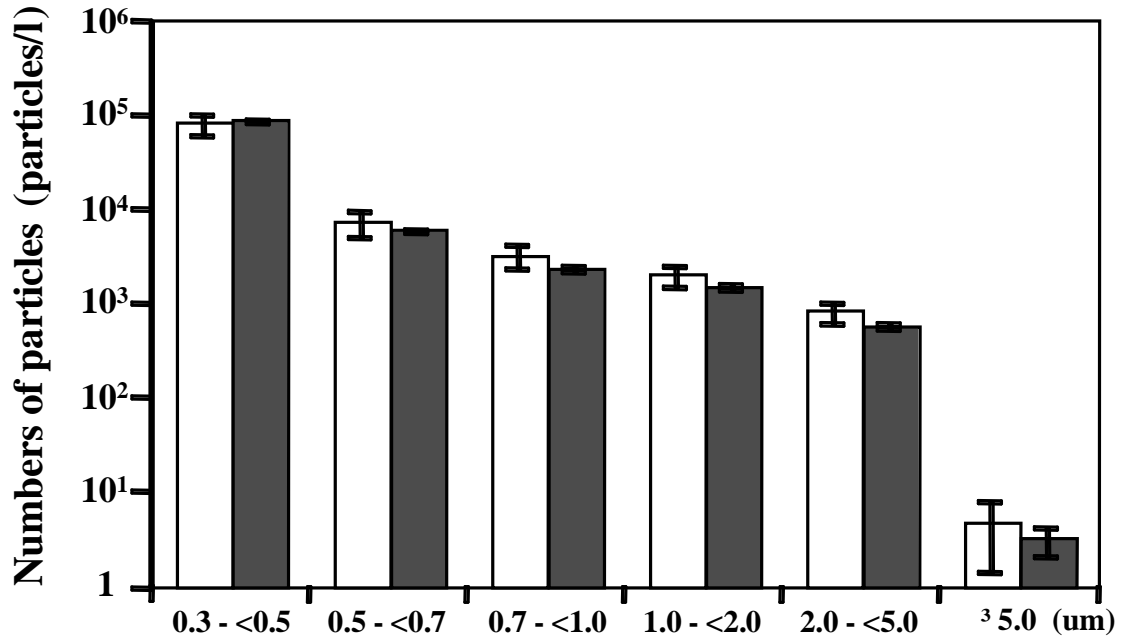
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Fig. 1 T. Maki et al.

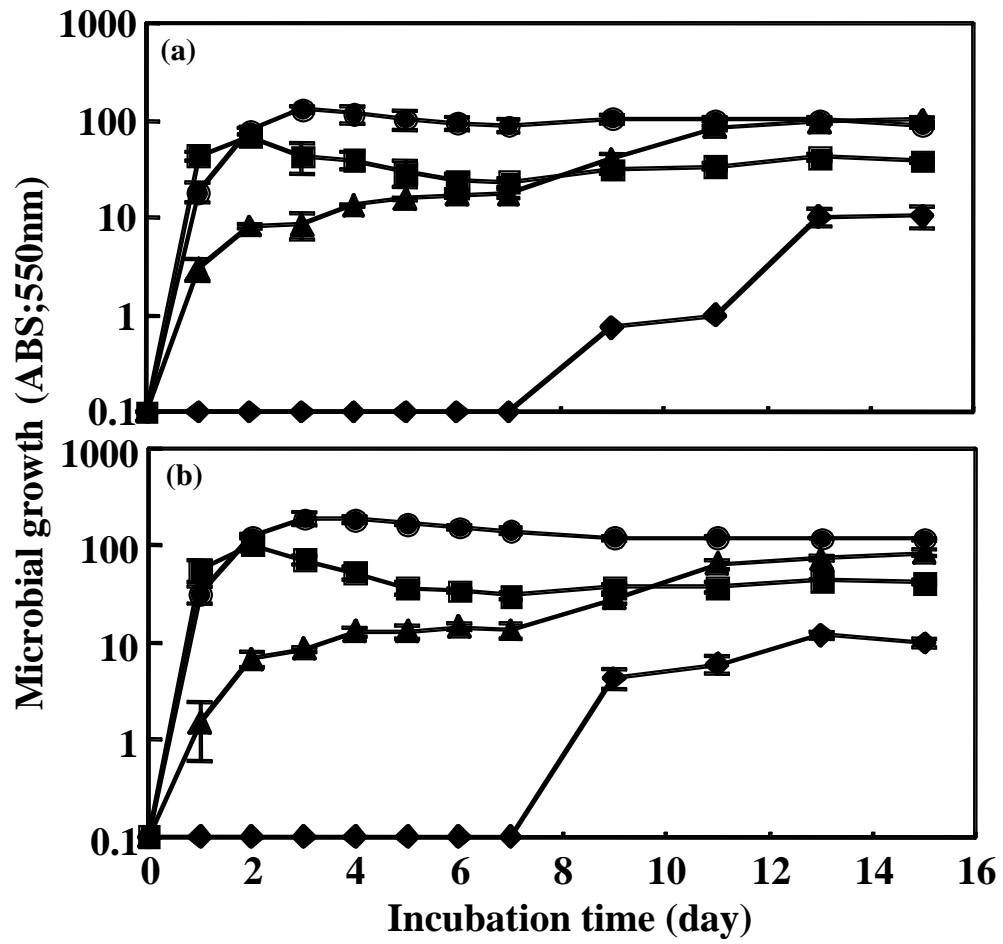
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Fig. 2 T. Maki et al.

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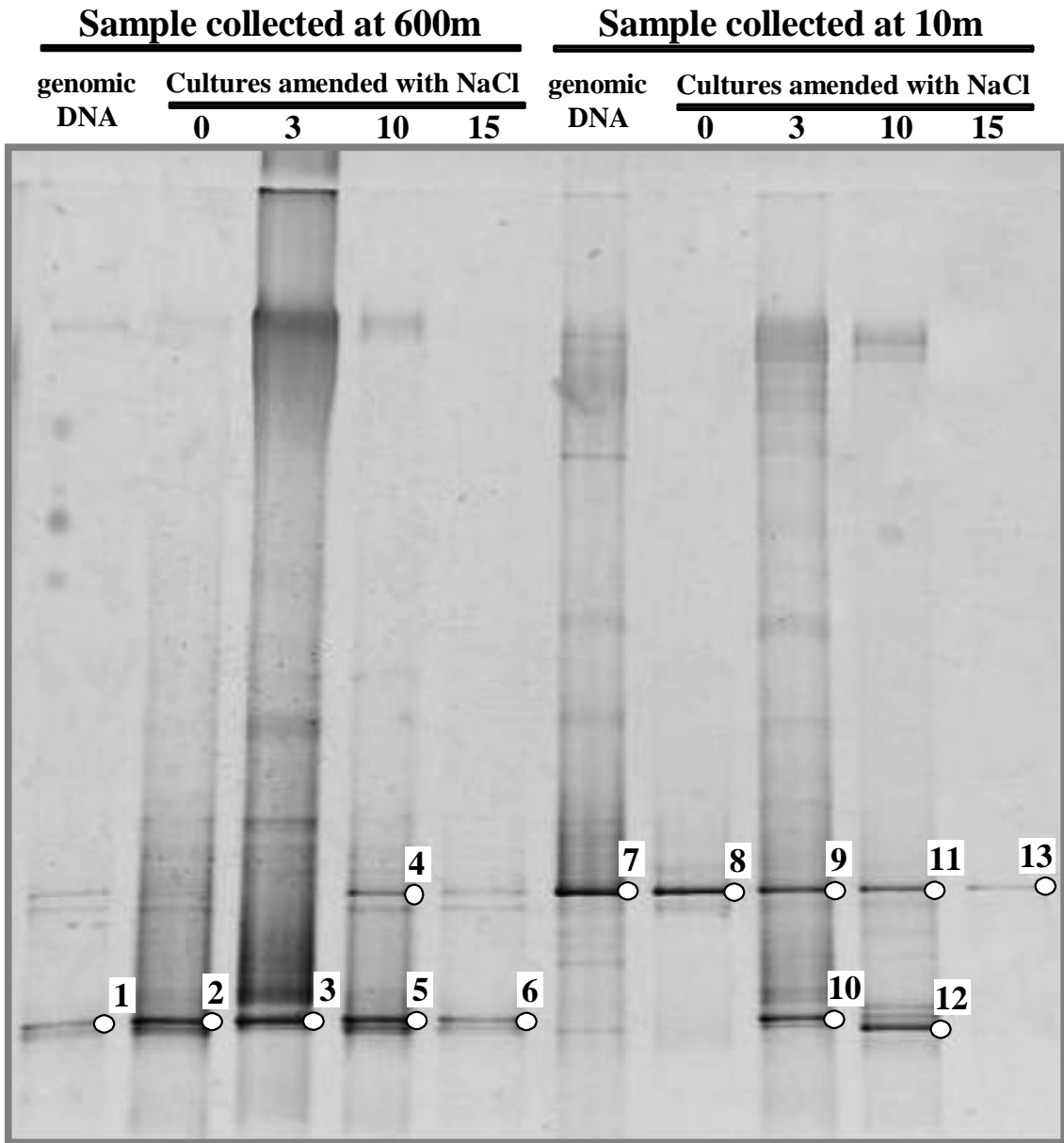


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Fig. 3 T. Maki et al.

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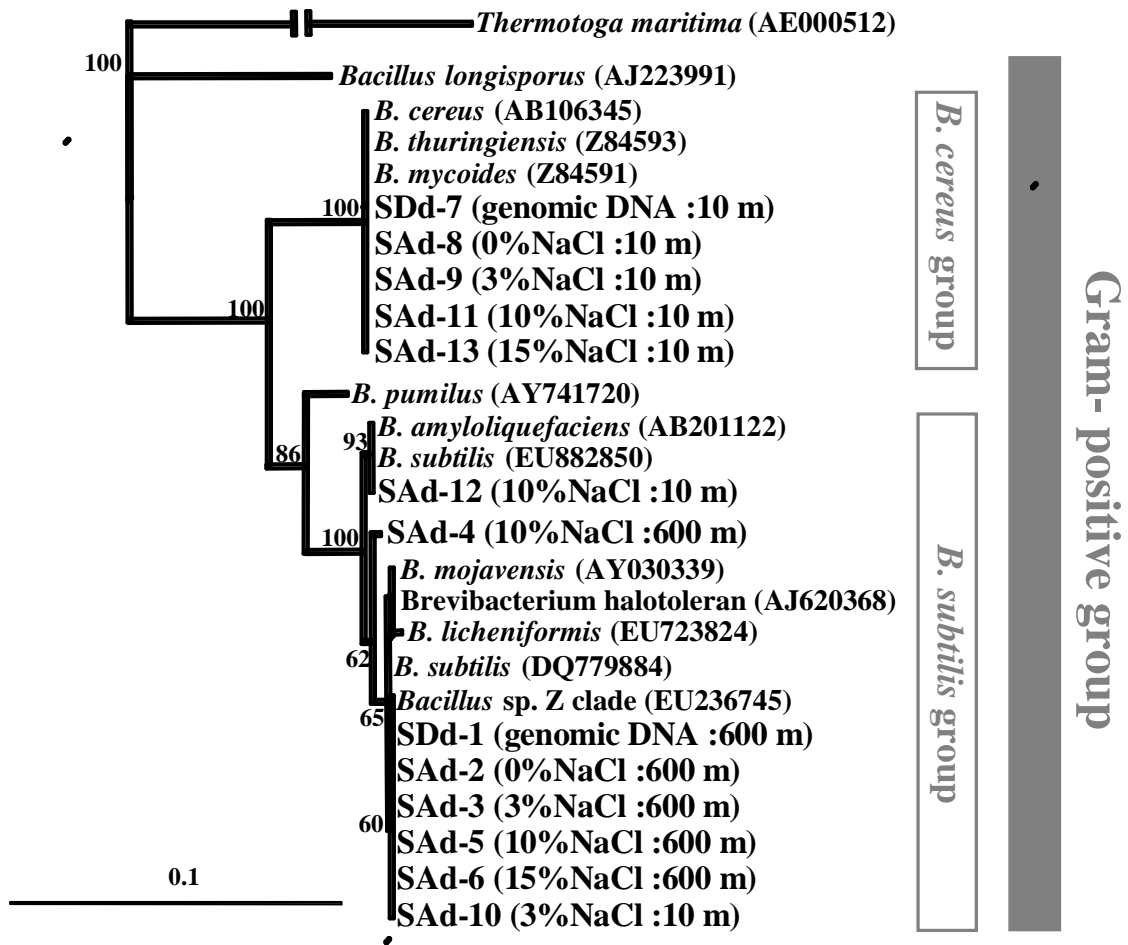
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Fig. 4 T. Maki et al.

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Fig. 5 T. Maki et al.