# NaCl-amendment assay targeting airborne bacteria in tropospheric bioaerosols transported by westerly wind over Noto Peninsula



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Title:

**NaCl amendment assay targeting airborne bacteria in tropospheric bioaerosols transported by westerly wind over Noto Peninsula**

Running Title:

**Bacteria transported through the troposphere**

Authors:

Teruya Maki * [1], Fumihisa Kobayashi[1], Maromu Yamada[2], Hiroshi Hasegawa[1], and Yasunobu Iwasaka[3]

Affiliation of all authors:

1. College of Science and Engineering, Kanazawa University, Kakuma, Kanazawa, Ishikawa, 920-1192, Japan.
2. National Institute of Occupational Safety and Health, Japan, 6-21-1, Nagao, Tama-ku, Kawasaki, Tokyo, 214-8585, Japan.
3. Community Research Service Group, University of Shiga Prefecture, 2500 Yasakamachi, Hikoneshi, Shiga, 522-8533, Japan.

*Corresponding author:

Tel: +81-(0) 76-234-4793, Fax: +81-(0) 76-234-4800

E-mail: makiteru@t.kanazawa-u.ac.jp

**Abstract**

Bioaerosol particles including bacteria, fungi and virus are originated from marine and terrestrial environments. The airborne microorganisms are transported for long-distance through the free troposphere and are thought to influence the downwind ecosystems and human life. However, microbial communities in the free troposphere have not been understood in detail because the direct sampling of microbial cells at high altitude requires sophisticated sampling techniques. In this study, for the investigation of microbial species compositions in the free troposphere, air sampling using an aircraft was performed over the Noto Peninsula in Japan, where the tropospheric winds carry aerosol particles from continental areas. Two air samples were collected at 3000 m on March 27th, 2010, when air mass was carried from the Gobi Desert to Japan area. Microorganisms from one air sample grew in culture media containing up to 15% NaCl, suggesting that halotolerant bacteria maintain their viabilities in the free troposphere. DGGE analysis revealed that the amended cultures were dominated by *Bacillus subtilis*, and the isolates obtained from the amended cultures were identical to *B. subtilis*. Furthermore, the 16S rDNA clone library (culture independent survey) of the other air sample grew was composed of three phylotypes belonging to *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* with the sequences of *Firmicutes* phylotype corresponding to that of the cultured *B. subtilis* sequence. Microscopic observation using FISH method indicated that *B. subtilis* particles occupied 80% of total eubacterial particles on the mineral particles. The halotolerant bacteria identical to *B. subtilis* would dominate at high altitudes over Noto Peninsula where the prevailing westerly wind was blowing.

**Key words:** Kosa, Asian dust, bioaerosol, halotolerant bacteria, free troposphere, atmosphere

## Introduction

Bioaerosol particles, which include microorganisms, are also transported from the continental areas to downwind regions through the free troposphere (Iwasaka et al. 2009; Prospero et al. 2005). Microorganisms in bioaerosols are significantly abundant in the organic carbon fraction of aerosol particles in the atmosphere and can remain viable in the free troposphere under extended UV exposure, low-moisture levels, and extremely oligotrophic conditions (Jones and Harrison 2004). In particular, the long-range transport of microorganisms by Asian dust events plays an important role in microbial dispersal and has significant impact on ecosystems, human health, and agricultural productivity in downwind areas (Jaenicke 2005). Ichinose et al. demonstrated that some microorganisms associated with Asian dust mineral particles increase allergen burden, with negative effects on human health, such as increased incidence of asthma (Ichinose et al. 2005). Moreover, bioaerosol particles are thought to influence atmospheric processes by participating in atmospheric chemistry and cloud formation. Microorganisms in the atmosphere are also known to act as ice nuclei and cloud condensation nuclei affecting ice-cloud processes (Pratt et al. 2009).

Microbial species composition of the atmosphere requires investigation to understand the characteristics of microbial communities that are transported for long distances and influence downwind ecosystems. In previous studies, aerosol sampling, using a balloon (Kobayashi et al. 2007) and a tower (Li et al. 2010) at altitudes ranging from 200m to 800m demonstrated that bioaerosols are composed of several species of bacteria. In particular, members of the genus *Bacillus* were associated with Kosa

mineral particles collected at high altitudes in downwind area during Kosa events (Maki et al. 2010). It has been reported that airborne microbial communities at ground level in Asian regions change significantly in species composition and abundance depending on Kosa events (Hara and Zhang 2012). The *Fermicutes* group mainly including *Bacillus* sp. was reported to dominate in the ground surface air during Kosa events (Jeon et al. 2011).

Viable microorganisms in troposphere are expected to maintain their ability to withstand desiccation, extreme temperatures, oxygen limitations, or extended UV exposure (Alan & Harrison 2004). Halotolerant bacteria are known to tolerant to these environmental stressors as well as high salinity (Russell 1989), and to be typical of bioaerosols that are transported across hundreds to thousands of kilometers (Yukimura et al. 2009). Some halotolerant bacteria isolated from sand dunes in the Gobi Desert were belonging to the genus *Bacillus*, which includes species such as *B. subtilis* and identical to bacterial species isolated in Higashi-Hiroshima, Japan, indicating the possibility of their long-range transport (Hua et al., 2007). An experimental design facilitating halotolerant bacterial activities in bioaerosol samples is expected to be useful for analyzing the atmospheric microorganisms. In fact, halotolerant bacteria belonging to the genus *Bacillus* have been detected from bioaerosol particles collected at an altitude of 800 m in the Kosa source area, Dunhang City (Maki et al. 2008). However, few reports have directly investigated *Bacillus* species at high altitudes, such as the free troposphere, where long-range transported aerosol is abundant, because the direct sampling of microbial cells in the troposphere requires sophisticated sampling techniques.

In this study, two samples were collected at altitudes of 3000 m above the north coast of Noto Peninsula of Japan on March 27th, 2010, when air mass was carried through the Gobi Desert to Japan area. The viabilities of halotolerant bacterial communities in one air sample were evaluated by NaCl-amendment assays using culture media with different NaCl concentrations. Bacterial species compositions in the other air sample and the NaCl amended cultures were determined using culture dependent and independent techniques targeting bacterial 16S rRNA genes.

## Materials and Methods

### Sampling

Aerosol sampling using an aircraft was performed over the north coast of the Noto Peninsula of Japan using an aircraft from 14:50 to 16:50 on March 27th, 2010. A sampling course is from Suzu City (37.5°N, 137.4°E) to the sea area (37.5°N, 136.4°E) (Fig. 1). Aerosol compositions in the troposhere over the Noto Peninsula are often influenced by aerosol particles that tropospheric winds carry from continental areas. The backward trajectories were calculated from the NOAA Hybrid Single Particle Lagrange Integrated Trajectory (HYSPLIT) model (http://www.arl.noaa.gov/HYSPLIT.php). Meteorological conditions during the sampling periods were estimated based on the meteorological data provided by the Wajima Meteorological Observatory of the Japan Meteorological Agency. Particle number concentrations were measured with an optical particle counter (KR-12A: RION CO., Ltd., Tokyo, Japan) during the aerosol sampling.

The air samples were collected at 3000 m above the ground using the aircraft that

had a 25-mm-diameter hole on the top (Kobayashi et al. 2011). Sterilized sampling tubes, 1.5 m in length, were inserted into the hole with the edges of the two tubes reaching the outside. The other edges of the two tubes were connected to the sterilized filter holders (In-Line Filter Holder, 47 mm; Millipore, Tokyo, Japan) in the sampling devices. Air samples (1400 l) were collected on two sterilized polycarbonate filters (0.22 μm pore size; Whatman, Tokyo, Japan) for 2 h. In total, two filters with air samples were obtained for each sampling period. Within 2 h of sampling, the aerosol particles were washed off the filters by shaking with 10 ml of sterilized water containing 0.9% (w/v) NaCl. The solution thus obtained from one filter was used to determine particle density by microscopic observation and was used as a cultivation spike in media containing different NaCl concentrations for investigating the viability of halotolerant bacteria. The solution obtained from the other filter was used to estimate bacterial species composition by PCR-DGGE analysis and clone-library analysis targeting 16S rDNA.

**Determination of particle abundance by microscopic observation**

The solution obtained after washing (2 ml) was fixed with paraformaldehyde solution at a final concentration of 1%. The samples were stained with DAPI (4’,6-diamidino-2-phenylindole) at a final concentration of 0.5 μg/ml for 15 min and filtered through a 0.22-μm pore-size polycarbonate filter (Whatman) stained with Sudan Black (Russell et al. 1974). After the filter was placed on a slide on a drop of low-fluorescence immersion oil, a drop of oil was added and a cover was placed. Particles on the filters were observed using an epifluorescence microscope (Olympus,

Tokyo, Japan) with a UV excitation system. After a filter transect was scanned, the numbers of mineral particles, yellow particles and bacterial cells on the filter transect were counted. The particle numbers counted on 20 filter transects were used for the calculation of particle concentrations. The detection limit of aerosol particles was below $5 \times 10^2$ particles/liter air mass.

**Physiological experiments**

To evaluate viabilities of halotolerant bacteria in the air samples, 0.5 ml of the solution obtained after washing was inoculated into 19.5 ml of TS (Trypticase Soy Peptone) liquid medium (17 g trypticase peptone, 5 g phytone peptone, 2.5 g $K_2PO_4$, and 2.5 g glucose in 1 liter of pure water) with NaCl at final concentrations of 0%, 3%, 10%, or 15% (w/v). TS medium has often been used for detecting bacteria from air samples. Microbial growth was estimated every 2 days at 550-nm absorbance. After 12 days of incubation, the microbial cultures were used for isolating bacteria by culture technique and determining species diversity by PCR-DGGE analysis.

**Identification of bacterial isolates using 16S rRNA gene information**

The bacteria in the NaCl amended cultures were isolated using the spread-plate method. Ten µl of the culture was plated onto TS agar plates. After the bacterial isolates were incubated in the 10 ml of TS medium for 3 days, the bacterial cells were collected using the centrifugation of 20000 x g for 5 min. The bacterial cells were used for the extracting of genomic DNA (gDNA) using SDS, proteinase K, and lysozyme as described previously (Maki et al. 2008). The gDNA was purified by phenol-chloroform

extraction, chloroform extraction, and ethanol precipitation. Fragments of 16S rDNA (ca. 1450 bp) were amplified from the extracted gDNA by PCR using the following oligonucleotide primers: 27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′; and 1492R, 5′-GGY TAC CTT GTT ACG ACT T-3′ (Maidak et al. 1997). Thermal cycling was performed using a Program Temp Control System PC-700 under the following conditions: denaturation at 94°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min, for a total of 30 cycles. The PCR amplicons were purified by phenol-chloroform extraction and chloroform extraction followed by ethanol precipitation. The nucleotide sequences were determined using a Dye Deoxy$^{TM}$ Terminator Cycle Sequencing Kit (ABI, CA, USA) and a DNA autosequencing system (ABI, Model 373A) according to the manufacturer's recommended protocol. The primers 27F and 1492R were used as the sequencing primer. The determined sequences were compared with DDBJ (DNA Data Bank of Japan) database and a phylogenetic tree was constructed according to the procedures as describes (Saitou and Nei 1987).

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

Filter-washing solutions (10 ml) of air samples and the solution (1 ml) of NaCl amended cultures were used for the extracting of gDNA. The gDNAs were extracted and purified as described above (Maki et al. 2008). A 16S rDNA region (ca. 550 bp) of the extracted gDNA was amplified by PCR using the following oligonucleotide primers: F341-GC, 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3′; and R907, 5′-CCG TCA ATT CCT TTR AGT TT-3′ (Muyzer et al. 1993). For each PCR reaction, 10 ng of the extracted DNA was

186 added to a PCR mastermix (20 µl) containing 2 µmol/l of dNTPs (TaKaRa, Ohtsu,
187 Japan), 2 nmol/l of each primer, and 1 U of Taq DNA polymerase (TaKaRa). Thermal
188 cycling was performed using a Program Temp Control System PC-700 (ASTEC,
189 Fukuoka, Japan) with the following thermal cycling program: a hot-start denaturing step
190 of 5 min at 94°C; 20 cycles of 1 min at 94°C, 1 min at 65−55°C (touchdown
191 −1.0°C/2cycles), and 3 min at 72°C; 15 cycles of 1 min at 94°C, 1 min at 55°C, and 3
192 min at 72°C; and a final extension step of 10 min at 72°C. Amplification was verified
193 by agarose (1.5% w/v) gel electrophoresis.
194 DGGE analysis was performed with 6% acrylamide gels containing a linear
195 gradient of denaturant from 40% to 60% [100% denaturant consisted of 7 mol/l of urea
196 and 40% (v/v) formamide]. Electrophoresis was performed at 60°C and 90 V for 16 h in
197 a 1 × TAE buffer with an electrophoresis system (AE-6290; ATTA, Tokyo, Japan).
198 After electrophoresis, the gels were stained with SYBR Gold and scanned in a
199 Printgraph (AE-6933FXCF; ATTA). Several bands on the gels were excised for
200 sequencing. The excised gel pieces were transferred to PCR tubes, and the PCR
201 amplicons (ca. 550 bp) were purified by phenol-chloroform extraction and chloroform
202 extraction followed by ethanol precipitation. The nucleotide sequences were determined
203 using a Dye Deoxy™ Terminator Cycle Sequencing Kit (ABI, CA, USA) and a DNA
204 autosequencing system (Model 373A; ABI) according to the manufacturer's
205 recommended protocol. Primer F-341 without a GC clamp was used as the sequencing
206 primer. The determined sequences were compared with DDBJ database and
207 phylogeneticaly analyzed as described (Saitou and Nei 1987).
208

**Clone libraries of bacterial 16S rDNA**

The gDNAs directly extracted from the filter-washed solutions were used for amplifying fragments of 16S rDNA (ca. 1450 bp) by PCR using the primers 27F and 1492R. Thermal cycling was performed using a Program Temp Control System PC-700 under the following conditions: denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 2 min, for a total of 30 cycles. The PCR amplicons of 16S rDNA fragments were cloned into *Escherichia coli* using a commercially prepared vector with a TA Cloning Kit (Invitrogen, CA, USA) according to the manufacturer's protocol. More than 60 clones were obtained and the sequences were determined as described above, except that the sequence primer was replaced with the M13 forward and reverse primers. The determined sequences were compared with DDBJ database and phylogeneticaly analyzed as described (Saitou and Nei 1987).

**Fluorescence *in situ* hybridization (FISH)**

For analysis of the bacterial distribution, the bacteria attached on the Kosa mineral particles were stained and observed by the FISH technique with nucleotide probes (Maki et al. 2004). The oligonucleotide probe PB-BS 28 was used to detect the ribosomal RNA of *Bacillus subtilis* (Haruta et al. 2002). In addition, the probes Eub338R for eubacteria (Amann et al. 1990) and non-Eub were also used as a positive control and a negative control, respectively. Sequences of the probes PB-BS28, Eub338R, and non-Eub were 5′-ACA GAT TTG TGG GAT TGG CT-3′, 5′-GCT GCC TCC CGT AGG AGT-3′, and 5′-CGA CGG AGG GCA TCC TCA-3′. For the activation of microbial cells, the filter solution (6 ml) was incubated with TS liquid

medium (1 ml) addition for 1 hour. The filter wash solutions were incubated with 10mg/l lysozyme solution for 5 min and fixed with paraformardehyde solution (final concentration of 4%) in PBS (200 mM sodium phosphate buffer, pH 7.4) for 3 hr at room temperature. The aerosol particles in 2ml wash solutions were bound on a nuclepore filter (0.2 μm of pore size). Totally, 3 nuclepore filters were prepared and each nuclepore filter was put into an eppendorf tube, and washed sequentially with 50, 80 and 100 % ethanol for 1 min. Then, 20 μl of hybridization solution (30 % formamide, 0.9 M NaCl, 0.1 % sodium dodecyl sulfate, 20 mM Tris buffer, pH 7.2) was added to each sample on the nuclepore filter. Following the preincubation at 30°C for 30 min, 9 μl of hybridization solution containing 2.5 μg of fluorescence isothiocyanate (FITC)-labeled probe was added. The mineral particles were hybridized at 30°C for 6 hr in a water bath, and washed with 20-40 μl of hybridization solution twice at 30°C for 20min.

After hybridization and washing, the nuclepore filter with the mineral particles was placed on a filtering device, and rinsed with distilled water. Subsequently, the filter was observed under an epifluorescence microscope (Olympus Co., Tokyo, Japan) equipped with the dichroic mirror system for FITC (excitation wavelength, 465-495nm; dichroic mirror, 505nm). Photomicrographs were taken with color reversal 400nm film (FUJIFILM, Tokyo, Japan). After a filter transect was scanned, the bacterial particles on the filter transect were counted.

**Accession numbers**

The DDBJ accession numbers for the 16S rDNA sequences determined in this study

255 are from AB740155 to AB740159 and from AB740968 to AB740970.

256

257

258 **Results**

259

260 **Environmental factors**

261 Analysis of air-mass backward trajectories revealed that the air mass of March 27th
262 2010 was carried from the Gobi Desert area and passed over the industrial area in China
263 and across the Sea of Japan (Fig. 2). During sampling period, observed weather
264 condition at 3000 m was a clear sky and clouds coved over the ground area. The mean
265 temperature at 3000 m was -16.4 °C. According to the Wajima Meteorological
266 Observatory of the Japan Meteorological Agency, westerly winds of 18 m/s were
267 recorded at 700 hPa (about 3000 m above sea level) over the north coast of Noto
268 Peninsula at 9:00 p.m. (12:00 UTC) on March 27th, 2010.

269 When the aerosol particles in air samples collected at 3000m were observed using
270 epifluorescence microscopic observation using DAPI staining, the air samples of March
271 27th included mineral particles at concentrations of 1,050 ± 790 particles/l, and yellow
272 fluorescent particles were detected at concentrations of 1,930 ± 700 particles/l (Table 1).
273 The total density of bacterial cells associated with aerosol particles was 2,280 ± 830
274 particles/l. When particle densities at 3000m were measured using a particle counters,
275 particles between 0.3 - 0.5 μm of March 27th showed high concentrations of more than
276 45,000 particles/l and made up about 85% of the total number of particles (Table 1). In
277 addition, the air mass of March 27th included the particles between 0.3 - 2.0 μm at

concentrations ranging from 430 particles/l to 4,900 particles/l, and relatively large particles >2.0 μm at a concentration of 121 particles/l.

**NaCl amended cultures**

When the air sample collected at 3000m was inoculated in TS liquid media containing different NaCl concentrations, microbial growth in the media containing 0%, 3%, and 10% NaCl rapidly increased to an absorbance of >95 (approximately $4 \times 10^7$ cells/ml) within 5 days of incubation and fluctuated between 51 and 420 during the experimental period (Fig. 3). Cultures amended with 15% NaCl began to show minimal microbial growth from the 4th day, and the absorbance gradually increased to approximately 25 over the experimental period. These results indicated that microorganisms that were tolerant to NaCl concentrations of up to 15% maintained their viability in the air sample collected on March 27th.

Colonies on the agar plates on which the NaCl amended cultures were spread were picked up judging by colony formation and colors. Consequently, total five isolates were obtained from each NaCl amended cultures including 0%, 3%, 10%, or 15% NaCl. The full sequences of 16S rDNA (ca. 1450) of the 4 isolates belonged to the group of *B. subtilis* in *Firmicutes* and indicated high similarities at >99.9% (Table 2).

**DGGE analysis of bacterial communities**

When the bacterial species composition of the air sample was determined using PCR-DGGE analysis, the gDNA extracted directly from the air sample and from the NaCl-amended cultures showed different banding patterns, with three DGGE bands and

301 one band, respectively (Fig. 4). The DGGE bands of cultures amended with 0%, 3%,
302 10%, and 15% NaCl each showed a single dominant band (SAd-2, SAd-3, SAd-4, and
303 SAd-5) at identical positions. These single dominant bands were identical to one
304 (SDd-1) of the three bands obtained from gDNA extracted directly from the sample.
305 The 16S rDNA sequences of the dominant bands (SDd-1, SAd-2, SAd-3, SAd-4, and
306 SAd-5) yielded a single phylotype that had 100% similarity to that of *B. subtilis* (Table
307 2). This indicated that a single bacterial species was common to the cultures at all NaCl
308 concentrations. The remaining two bands (SDd-6 and SDd-7) were specific to the
309 gDNA extracted directly from the air sample. The SDd-6 sequence had 98.5% similarity
310 to that of *Rhodanobacter terrae* (Table 2). The SDd-7 sequence belonged to
311 *Bacteroidetes* and had 88.8% similarity to that of *Owenweeksia hongkongensis*,
312 suggesting that the phylotype including SDd-7 was a novel bacterial species. These
313 results indicated that members of three phylotypes were abundant in the air sample and
314 that the one phylotype dominant in the troposphere could grow by enrichment culture.
315
316 **Comparison of 16S rDNA clones**
317 16S rDNA fragments (ca. 1450bp) in the air sample were amplified by PCR with
318 primers targeting eubacterial 16S rDNA. The PCR amplicons were cloned into *E. coli*,
319 and a total of 65 clones including eubacterial 16S rDNA fragments were obtained from
320 the air sample. Sequences of the 16S rDNA clones showed that the bacterial populations
321 were divided into 3 phylotypes defined as sequences with >98% sequence similarity
322 (Table 2). The majority of phylotypes were affiliated with *Firmicutes, Bacteroidetes,*
323 and *Gammaproteobacteria* lineages that are typically well represented in 16S rDNA

clone libraries generated from terrestrial and marine environments (Table 2). In particular, sequences belonging to *Firmicutes* accounted for 85% of total clones. All *Firmicutes* sequences fell into a single phylotype that was closely related to *B. subtilis* with high similarities of >99.7% and was identical to the sequence of the dominant DGGE bands (SDd-1, SAd-2, SAd-3, SAd-4, and SAd-5) and the sequence of isolates obtained from NaCl amended cultures (Fig. 5). Another phylotype, including four clones belonging to *Bacteroidetes*, was related to *O. hongkongenesis* at a low similarity between 88.3% and 88.4%, and was >98.6% identical to the sequence of DGGE band (SDd-7). The one remaining clone belonged to *Xanthomonadaceae* in *Proteobacteria* and was closely related to *Pseudoxanthomonas byssovorax* with a similarity of 93.6%.

**Whole-mineral particle *in situ* hybridization targeting bacterial cells**

Epifluorescence microscopy after whole-particles *in situ* hybridization of mineral particles collected at 3000m revealed that the probes PB-BS 28 for *B. subtilis* and Eub338R for eubacteria bound to the small particles on the surfaces of mineral particles (Fig. 6). Particles bounded with the probe non-Eub (negative control) were not observed (data not shown). The signals by PB-BS 28 occupied approximately 80% of small particles among the all small particles detected by Eub 338 for eubacteria (Table 4), suggesting that the nucleotides originated from *B. subtilis* cells dominated on the mineral particles.

**Discussion**

347 The westerly wind at high altitudes over Asian region is known to carry Kosa
348 mineral particles associated with microorganisms across hundreds and thousands of
349 kilometers, and these airborne microorganisms are dispersed around the Asian
350 downwind areas through the free troposphere (Griffin et al. 2003; Iwasaka et al. 2009).
351 The air mass over Noto Peninsula on March 27th, 2010, was carried from the
352 continental desert areas (Fig. 2) and had high amounts of aerosols and included
353 significant amount of mineral, yellow and bacterial particles (Table 1). DAPI
354 yellow-fluorescing particles have been reported to resemble organic materials
355 originating from microbial cell components such as proteins (Mostajir et al. 1995). The
356 Japan Meteorological Agency reported westerly winds of 18 m/s at 3000 m above the
357 ground during the sampling period. Furthermore, SYNOP (surface synoptic
358 observations) database indicated that dust events occurred at several sites in the
359 continental desert areas for 3 days before the sampling dates. During the spring and
360 summer seasons, the prevailing westerly winds is thought to constantly carry dust
361 particles throughout the free troposphere and cause the weak Kosa at a height of 4000 m
362 over East Asia (Iwasaka et al. 1988; Matsuki et al. 2003). Kosa events have been
363 reported to increase the number of airborne microorganisms on ground surfaces in
364 correspondence with the amount of mineral particles (Hara and Zhang 2012). During
365 the March 27th sampling period, the prevailing westerly wind is believed to carry
366 aerosol particles from continental areas to high altitudes above Noto Peninsula.
367 The NaCl amendment culture demonstrated that the air sample collected at 3000m
368 on March 27th showed significant microbial growth in the culture media including up to
369 15% NaCl (Fig. 3). Halotolerant bacteria are known to survive in extreme environments

370 through resistance to several stressors, such as desiccation, UV irradiation, extreme
371 temperatures, oxygen limitation, and high salinity (Russell 1989). Halotolerant bacteria
372 have been isolated from the ice cores of Greenland, suggesting the long-range transport
373 of the bacteria by dust events (Yukimura et al. 2009). The bacterial communities in the
374 NaCl amended cultures and the halotolerant isolates obtained from the cultures were
375 mainly composed of *B. subtilis* (Table 2). *Bacillus* spp. are known to form endospores
376 that are resistant to environmental stressors and that enhance their survival in the
377 atmosphere (Nicholson et al. 2000). Presumably, *B. subtilis* can resistant to high salinity
378 maintained its viability in the free troposphere during the sampling period, when the
379 weak Kosa is thought to occur. Halotolerant bacteria that are resistant to atmospheric
380 stressors would maintain their viabilities and be selected among entire airborne bacteria,
381 originated from ground area.

382 The sequences of *B. subtilis* growing in the NaCl-amended cultures were identical
383 to a sequence detected from gDNA collected from the March 27th air sample and were
384 abundant in the 16S rDNA clone library obtained from the sample (Table 2, Fig. 5).
385 FISH technique revealed that *B. subtilis* cells occupied approximately 80 % of total
386 cells of activated microorganisms (Table 3). The species composition of cultured
387 isolates obtained from natural environments are often different from the diverse
388 bacterial lineages detected using culture-independent techniques (Maron et al. 2005),
389 because 99% of environmental bacteria can not be cultivated by traditional methods
390 (Olsen and Bakken 1987). In contrast, the clone libraries obtained from Antarctic and
391 Arctic pack ice samples revealed strong phylotype overlap with cultivated isolates
392 (Brinkmeyer et al. 2003). In the air sample collected at 3000 m on March 27th, *B.*

393 *subtilis* would be a dominant species, and the viability of *B. subtilis* could be amended
394 using culture techniques.
395 Although this sampling was performed during a single period, two air samples
396 commonly included *B. subtilis*, indicating the high possibility that this species was
397 transported by the westerly wind. The members of *B. subtilis* group including
398 halotolerant bacteria were dominantly associated with dust mineral particles collected at
399 altitudes some hundreds of meters above the Taklamakan Desert (Maki et al. 2008) and
400 Suzu City during Kosa events (Maki et al. 2010). In the snow cover of Mt. Tateyama
401 accumulating aerosols with snow fall during the winter and spring seasons, the snow
402 layer that included Kosa mineral particles contained halotolerant bacteria identified as
403 the *B. subtilis* group but layers without dust particles did not contain *B. subtilis* (Maki et
404 al. 2011). Species related to *B. subtilis* were isolated from sand samples of the Gobi
405 Desert area (Hua et al. 2007) and reported to dominate in the surface air of Saul City
406 during Kosa events (Jeon et al. 2011). Therefore, *B. subtilis* in the air sample was
407 possibly transported with dust mineral particles from the continental desert area.
408 Although members of *B. subtilis* group are most often thought to be
409 non-pathogenic and clinical contaminants, they are considered to be serious nosocomial
410 bacteria infecting injured persons (Richard et al. 1988) and immunosuppressed patients
411 (Velasco et al. 1992). In contrast, the *B. subtilis* group included antagonists, which
412 suppress the pathogenic diseases of plants (Alabouvette et al. 1996) and cultured shrimp
413 (Banerjee et al. 2007). Furthermore, some strains of *B. subtilis* have been used for the
414 production of Japanese health foods such as *natto* (Ashiuchi et al. 1998). Communities
415 of the *B. subtilis* group are reported to degrade organic matters, thus contributing to the

416 carbon cycle in terrestrial environments (Das and Mukherjee 2007). Therefore, the
417 atmospheric transports of the *B. subtilis* group might have negative and positive
418 influence on human societies and environmental ecosystems. There is a possibility that
419 atmospheric transport of the *B. subtilis* group influences several aspects of human
420 societies and environmental ecosystems in Asian regions.

421 FISH technique revealed that the approximately 20% of eubacterial cells would be
422 composed of minor species except for *B. subtilis* (Table 4). *Bacteroidetes* sequences
423 detected from the clone library were identical to a DGGE band of gDNA extracted
424 directly from the air sample (Table 2). Moreover, the clone library of air sample also
425 included members of *Proteobacteria* belonging to the *Xanthomonadaceae* group (Fig.
426 5). Some species belonging to *Bacteroidetes* and *Proteobacteria* are expected to be
427 transported by the westerly wind. The eubacterial cells bounded with the probe
428 Eub338R showed higher concentrations than the bacteria identified with DAPI did.
429 FISH technique was reported to identify just 40%-80% of bacteria with nucleotide
430 probes in respect of total bacteria identified with DAPI, because the hybridization
431 probes target to only ribosomal RNA (Lew et al. 2010). In this study, the incubation
432 with TS medium addition would induce the proliferation of bacterial cells on mineral
433 particles. The minor bacterial species may also maintain their viabilities in atmosphere,
434 and the bacterial concentrations estimated by FISH were overestimated.

435

436 **Conclusion**

437

This study reported the bacterial communities in the free troposphere over Noto Peninsula (altitudes of 3000 m) when the air masses were transported from continental areas by westerly wind. Halotolerant bacteria belonging to the *B. subtilis* group would maintain their viabilities and dominated in the free troposphere over Noto Peninsula. There are possibilities that atmospheric stressors selected halotolerant bacteria among several species of airborne bacteria originated from ground area, and that the westerly wind carried *B. subtilis* through the free troposphere. In addition, there were some species of unculturable bacteria belonging to *Proteobacteria* and *Bacteroides* in the free troposphere. In future, more clone libraries of microbial communities obtained by several sampling at high are required to determine the origin region in continental areas or sea areas. Moreover, several bioaerosol samples can be compared for establishing a database of microbial communities transported for long distance to Japan by tropospheric winds.

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595 **Figure Legends**

596

597 Fig. 1 A route of aircraft sampling (solid line) from Suzu City (White circle; 37.5°N,
598 137.4°E) to the sea area (Black circle; 37.5°N, 136.4°E) during the sampling period
599 from 14:50 to 16:50 on March 27th, 2010.

600

601 Fig. 2 Three-day backward trajectories of aerosols that arrived Suzu City on March 27th,
602 2010.

603

604 Fig. 3 Microbial growth of bioaerosol sample collected at altitudes of 3000 m on March
605 27th, 2010, in media containing NaCl at concentrations of 0 % (square), 3 % (circle), 10
606 % (triangle) and 15 % (diamond). All experiments were performed in five test tubes.

607

608 Fig. 4 DGGE profile (band patterns) of amplified 16S rDNA from genomic DNA
609 directly extracted from the air sample collected at 3000 m on March 27th, 2010, and
610 from the bacterial cultures of air sample collected at 3000 m, which were cultivated in
611 TS media containing 0 %, 3 %, 10 %, and 15 % NaCl. A 40 % (upper side) to 60 %
612 (lower side) denaturing gradient was used.

613

614 Fig. 5 Phylogenetic tree including the partial sequences of 16S rDNA amplicons
615 obtained from NaCl amended bacterial isolates, DGGE bands and 16S rDNA clones.
616 The tree was calculated from a dissimilarity matrix of a ca. 553-bp alignment (*E. coli*
617 numbering 372 to 900) using a neighbor-joining algorithm. Sequences of Szi series

were obtained from the bacterial isolates from the NaCl amended cultures. Sequences of SzDd and SzAd series were obtained from the DGGE bands of the NaCl-amended cultures and gDNA extracted directly from the air sample, respectively. SzDc-March series indicate sequences of the 16S rDNA clone library. The sample information and the accession number of each reference sequence are given in parentheses. Bootstrap values >50% (after 1,000 resamplings) are indicated on the branches.

Fig. 6 Photographs indicating whole-mineral particles *in situ* hybridization against bacterial particles attached on Kosa-mineral particles that are collected at 3000 m on March 27th, 2010. Kosa-mineral particles were hybridized and stained with three FITC-labeled probes, Eub338R (probe for eubacteria; a), and PB-BS28 (probe for *B. subtilis*; b). Arrows in the micrographs show outline of the bacterial cells detected by FITC-labeled probes. All photomicrographs were taken at a magnification of x1000. (scale bar shows 5μm).

Title:

**NaCl amendment assay targeting airborne bacteria in tropospheric bioaerosols transported by westerly wind over Noto Peninsula**

Running Title:

**Bacteria transported through the troposphere**

Authors:

Teruya Maki * [1], Fumihisa Kobayashi[1], Maromu Yamada[2], Hiroshi Hasegawa[1], and Yasunobu Iwasaka[3]

Affiliation of all authors:

1. College of Science and Engineering, Kanazawa University, Kakuma, Kanazawa, Ishikawa, 920-1192, Japan.
2. National Institute of Occupational Safety and Health, Japan, 6-21-1, Nagao, Tama-ku, Kawasaki, Tokyo, 214-8585, Japan.
3. Community Research Service Group, University of Shiga Prefecture, 2500 Yasakamachi, Hikoneshi, Shiga, 522-8533, Japan.

*Corresponding author:

Tel: +81-(0) 76-234-4793, Fax: +81-(0) 76-234-4800

E-mail: makiteru@t.kanazawa-u.ac.jp

**Abstract**

Bioaerosol particles including bacteria, fungi and virus are originated from marine and terrestrial environments. The airborne microorganisms are transported for long-distance through the free troposphere and are thought to influence the downwind ecosystems and human life. However, microbial communities in the free troposphere have not been understood in detail because the direct sampling of microbial cells at high altitude requires sophisticated sampling techniques. In this study, for the investigation of microbial species compositions in the free troposphere, air sampling using an aircraft was performed over the Noto Peninsula in Japan, where the tropospheric winds carry aerosol particles from continental areas. Two air samples were collected at 3000 m on March 27th, 2010, when air mass was carried from the Gobi Desert to Japan area. Microorganisms from one air sample grew in culture media containing up to 15% NaCl, suggesting that halotolerant bacteria maintain their viabilities in the free troposphere. DGGE analysis revealed that the amended cultures were dominated by *Bacillus subtilis*, and the isolates obtained from the amended cultures were identical to *B. subtilis*. Furthermore, the 16S rDNA clone library (culture independent survey) of the other air sample grew was composed of three phylotypes belonging to *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* with the sequences of *Firmicutes* phylotype corresponding to that of the cultured *B. subtilis* sequence. Microscopic observation using FISH method indicated that *B. subtilis* particles occupied 80% of total eubacterial particles on the mineral particles. The halotolerant bacteria identical to *B. subtilis* would dominate at high altitudes over Noto Peninsula where the prevailing westerly wind was blowing.

**Key words:** Kosa, Asian dust, bioaerosol, halotolerant bacteria, free troposphere, atmosphere

## Introduction

Bioaerosol particles, which include microorganisms, are also transported from the continental areas to downwind regions through the free troposphere (Iwasaka et al. 2009; Prospero et al. 2005). Microorganisms in bioaerosols are significantly abundant in the organic carbon fraction of aerosol particles in the atmosphere and can remain viable in the free troposphere under extended UV exposure, low-moisture levels, and extremely oligotrophic conditions (Jones and Harrison 2004). In particular, the long-range transport of microorganisms by Asian dust events plays an important role in microbial dispersal and has significant impact on ecosystems, human health, and agricultural productivity in downwind areas (Jaenicke 2005). Ichinose et al. demonstrated that some microorganisms associated with Asian dust mineral particles increase allergen burden, with negative effects on human health, such as increased incidence of asthma (Ichinose et al. 2005). Moreover, bioaerosol particles are thought to influence atmospheric processes by participating in atmospheric chemistry and cloud formation. Microorganisms in the atmosphere are also known to act as ice nuclei and cloud condensation nuclei affecting ice-cloud processes (Pratt et al. 2009).

Microbial species composition of the atmosphere requires investigation to understand the characteristics of microbial communities that are transported for long distances and influence downwind ecosystems. In previous studies, aerosol sampling, using a balloon (Kobayashi et al. 2007) and a tower (Li et al. 2010) at altitudes ranging from 200m to 800m demonstrated that bioaerosols are composed of several species of bacteria. In particular, members of the genus *Bacillus* were associated with Kosa

mineral particles collected at high altitudes in downwind area during Kosa events (Maki et al. 2010). It has been reported that airborne microbial communities at ground level in Asian regions change significantly in species composition and abundance depending on Kosa events (Hara and Zhang 2012). The *Fermicutes* group mainly including *Bacillus* sp. was reported to dominate in the ground surface air during Kosa events (Jeon et al. 2011).

Viable microorganisms in troposphere are expected to maintain their ability to withstand desiccation, extreme temperatures, oxygen limitations, or extended UV exposure (Alan & Harrison 2004). Halotolerant bacteria are known to tolerant to these environmental stressors as well as high salinity (Russell 1989), and to be typical of bioaerosols that are transported across hundreds to thousands of kilometers (Yukimura et al. 2009). Some halotolerant bacteria isolated from sand dunes in the Gobi Desert were belonging to the genus *Bacillus*, which includes species such as *B. subtilis* and identical to bacterial species isolated in Higashi-Hiroshima, Japan, indicating the possibility of their long-range transport (Hua et al., 2007). An experimental design facilitating halotolerant bacterial activities in bioaerosol samples is expected to be useful for analyzing the atmospheric microorganisms. In fact, halotolerant bacteria belonging to the genus *Bacillus* have been detected from bioaerosol particles collected at an altitude of 800 m in the Kosa source area, Dunhang City (Maki et al. 2008). However, few reports have directly investigated *Bacillus* species at high altitudes, such as the free troposphere, where long-range transported aerosol is abundant, because the direct sampling of microbial cells in the troposphere requires sophisticated sampling techniques.

In this study, two samples were collected at altitudes of 3000 m above the north coast of Noto Peninsula of Japan on March 27th, 2010, when air mass was carried through the Gobi Desert to Japan area. The viabilities of halotolerant bacterial communities in one air sample were evaluated by NaCl-amendment assays using culture media with different NaCl concentrations. Bacterial species compositions in the other air sample and the NaCl amended cultures were determined using culture dependent and independent techniques targeting bacterial 16S rRNA genes.

## Materials and Methods

### Sampling

Aerosol sampling using an aircraft was performed over the north coast of the Noto Peninsula of Japan using an aircraft from 14:50 to 16:50 on March 27th, 2010. A sampling course is from Suzu City (37.5°N, 137.4°E) to the sea area (37.5°N, 136.4°E) (Fig. 1). Aerosol compositions in the troposhere over the Noto Peninsula are often influenced by aerosol particles that tropospheric winds carry from continental areas. The backward trajectories were calculated from the NOAA Hybrid Single Particle Lagrange Integrated Trajectory (HYSPLIT) model (http://www.arl.noaa.gov/HYSPLIT.php). Meteorological conditions during the sampling periods were estimated based on the meteorological data provided by the Wajima Meteorological Observatory of the Japan Meteorological Agency. Particle number concentrations were measured with an optical particle counter (KR-12A: RION CO., Ltd., Tokyo, Japan) during the aerosol sampling.

The air samples were collected at 3000 m above the ground using the aircraft that

had a 25-mm-diameter hole on the top (Kobayashi et al. 2011). Sterilized sampling tubes, 1.5 m in length, were inserted into the hole with the edges of the two tubes reaching the outside. The other edges of the two tubes were connected to the sterilized filter holders (In-Line Filter Holder, 47 mm; Millipore, Tokyo, Japan) in the sampling devices. Air samples (1400 l) were collected on two sterilized polycarbonate filters (0.22 μm pore size; Whatman, Tokyo, Japan) for 2 h. In total, two filters with air samples were obtained for each sampling period. Within 2 h of sampling, the aerosol particles were washed off the filters by shaking with 10 ml of sterilized water containing 0.9% (w/v) NaCl. The solution thus obtained from one filter was used to determine particle density by microscopic observation and was used as a cultivation spike in media containing different NaCl concentrations for investigating the viability of halotolerant bacteria. The solution obtained from the other filter was used to estimate bacterial species composition by PCR-DGGE analysis and clone-library analysis targeting 16S rDNA.

**Determination of particle abundance by microscopic observation**

The solution obtained after washing (2 ml) was fixed with paraformaldehyde solution at a final concentration of 1%. The samples were stained with DAPI (4’,6-diamidino-2-phenylindole) at a final concentration of 0.5 μg/ml for 15 min and filtered through a 0.22-μm pore-size polycarbonate filter (Whatman) stained with Sudan Black (Russell et al. 1974). After the filter was placed on a slide on a drop of low-fluorescence immersion oil, a drop of oil was added and a cover was placed. Particles on the filters were observed using an epifluorescence microscope (Olympus,

Tokyo, Japan) with a UV excitation system. After a filter transect was scanned, the numbers of mineral particles, yellow particles and bacterial cells on the filter transect were counted. The particle numbers counted on 20 filter transects were used for the calculation of particle concentrations. The detection limit of aerosol particles was below $5 \times 10^2$ particles/liter air mass.

**Physiological experiments**

To evaluate viabilities of halotolerant bacteria in the air samples, 0.5 ml of the solution obtained after washing was inoculated into 19.5 ml of TS (Trypticase Soy Peptone) liquid medium (17 g trypticase peptone, 5 g phytone peptone, 2.5 g $K_2PO_4$, and 2.5 g glucose in 1 liter of pure water) with NaCl at final concentrations of 0%, 3%, 10%, or 15% (w/v). TS medium has often been used for detecting bacteria from air samples. Microbial growth was estimated every 2 days at 550-nm absorbance. After 12 days of incubation, the microbial cultures were used for isolating bacteria by culture technique and determining species diversity by PCR-DGGE analysis.

**Identification of bacterial isolates using 16S rRNA gene information**

The bacteria in the NaCl amended cultures were isolated using the spread-plate method. Ten µl of the culture was plated onto TS agar plates. After the bacterial isolates were incubated in the 10 ml of TS medium for 3 days, the bacterial cells were collected using the centrifugation of 20000 x g for 5 min. The bacterial cells were used for the extracting of genomic DNA (gDNA) using SDS, proteinase K, and lysozyme as described previously (Maki et al. 2008). The gDNA was purified by phenol-chloroform

extraction, chloroform extraction, and ethanol precipitation. Fragments of 16S rDNA (ca. 1450 bp) were amplified from the extracted gDNA by PCR using the following oligonucleotide primers: 27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′; and 1492R, 5′-GGY TAC CTT GTT ACG ACT T-3′ (Maidak et al. 1997). Thermal cycling was performed using a Program Temp Control System PC-700 under the following conditions: denaturation at 94°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min, for a total of 30 cycles. The PCR amplicons were purified by phenol-chloroform extraction and chloroform extraction followed by ethanol precipitation. The nucleotide sequences were determined using a Dye Deoxy$^{TM}$ Terminator Cycle Sequencing Kit (ABI, CA, USA) and a DNA autosequencing system (ABI, Model 373A) according to the manufacturer's recommended protocol. The primers 27F and 1492R were used as the sequencing primer. The determined sequences were compared with DDBJ (DNA Data Bank of Japan) database and a phylogenetic tree was constructed according to the procedures as describes (Saitou and Nei 1987).

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

Filter-washing solutions (10 ml) of air samples and the solution (1 ml) of NaCl amended cultures were used for the extracting of gDNA. The gDNAs were extracted and purified as described above (Maki et al. 2008). A 16S rDNA region (ca. 550 bp) of the extracted gDNA was amplified by PCR using the following oligonucleotide primers: F341-GC, 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3′; and R907, 5′-CCG TCA ATT CCT TTR AGT TT-3′ (Muyzer et al. 1993). For each PCR reaction, 10 ng of the extracted DNA was

186 added to a PCR mastermix (20 µl) containing 2 µmol/l of dNTPs (TaKaRa, Ohtsu,
187 Japan), 2 nmol/l of each primer, and 1 U of Taq DNA polymerase (TaKaRa). Thermal
188 cycling was performed using a Program Temp Control System PC-700 (ASTEC,
189 Fukuoka, Japan) with the following thermal cycling program: a hot-start denaturing step
190 of 5 min at 94°C; 20 cycles of 1 min at 94°C, 1 min at 65−55°C (touchdown
191 −1.0°C/2cycles), and 3 min at 72°C; 15 cycles of 1 min at 94°C, 1 min at 55°C, and 3
192 min at 72°C; and a final extension step of 10 min at 72°C. Amplification was verified
193 by agarose (1.5% w/v) gel electrophoresis.
194 DGGE analysis was performed with 6% acrylamide gels containing a linear
195 gradient of denaturant from 40% to 60% [100% denaturant consisted of 7 mol/l of urea
196 and 40% (v/v) formamide]. Electrophoresis was performed at 60°C and 90 V for 16 h in
197 a 1 × TAE buffer with an electrophoresis system (AE-6290; ATTA, Tokyo, Japan).
198 After electrophoresis, the gels were stained with SYBR Gold and scanned in a
199 Printgraph (AE-6933FXCF; ATTA). Several bands on the gels were excised for
200 sequencing. The excised gel pieces were transferred to PCR tubes, and the PCR
201 amplicons (ca. 550 bp) were purified by phenol-chloroform extraction and chloroform
202 extraction followed by ethanol precipitation. The nucleotide sequences were determined
203 using a Dye Deoxy$^{TM}$ Terminator Cycle Sequencing Kit (ABI, CA, USA) and a DNA
204 autosequencing system (Model 373A; ABI) according to the manufacturer's
205 recommended protocol. Primer F-341 without a GC clamp was used as the sequencing
206 primer. The determined sequences were compared with DDBJ database and
207 phylogeneticaly analyzed as described (Saitou and Nei 1987).
208

209 **Clone libraries of bacterial 16S rDNA**

210 The gDNAs directly extracted from the filter-washed solutions were used for
211 amplifying fragments of 16S rDNA (ca. 1450 bp) by PCR using the primers 27F and
212 1492R. Thermal cycling was performed using a Program Temp Control System PC-700
213 under the following conditions: denaturation at 94°C for 1 min, annealing at 54°C for 2
214 min, and extension at 72°C for 2 min, for a total of 30 cycles. The PCR amplicons of
215 16S rDNA fragments were cloned into *Escherichia coli* using a commercially prepared
216 vector with a TA Cloning Kit (Invitrogen, CA, USA) according to the manufacturer's
217 protocol. More than 60 clones were obtained and the sequences were determined as
218 described above, except that the sequence primer was replaced with the M13 forward
219 and reverse primers. The determined sequences were compared with DDBJ database
220 and phylogeneticaly analyzed as described (Saitou and Nei 1987).

221

222 **Fluorescence *in situ* hybridization (FISH)**

223 For analysis of the bacterial distribution, the bacteria attached on the Kosa mineral
224 particles were stained and observed by the FISH technique with nucleotide probes
225 (Maki et al. 2004). The oligonucleotide probe PB-BS 28 was used to detect the
226 ribosomal RNA of *Bacillus subtilis* (Haruta et al. 2002). In addition, the probes
227 Eub338R for eubacteria (Amann et al. 1990) and non-Eub were also used as a positive
228 control and a negative control, respectively. Sequences of the probes PB-BS28,
229 Eub338R, and non-Eub were 5′-ACA GAT TTG TGG GAT TGG CT-3′, 5′-GCT GCC
230 TCC CGT AGG AGT-3′, and 5′-CGA CGG AGG GCA TCC TCA-3′. For the
231 activation of microbial cells, the filter solution (6 ml) was incubated with TS liquid

medium (1 ml) addition for 1 hour. The filter wash solutions were incubated with 10mg/l lysozyme solution for 5 min and fixed with paraformardehyde solution (final concentration of 4%) in PBS (200 mM sodium phosphate buffer, pH 7.4) for 3 hr at room temperature. The aerosol particles in 2ml wash solutions were bound on a nuclepore filter (0.2 μm of pore size). Totally, 3 nuclepore filters were prepared and each nuclepore filter was put into an eppendorf tube, and washed sequentially with 50, 80 and 100 % ethanol for 1 min. Then, 20 μl of hybridization solution (30 % formamide, 0.9 M NaCl, 0.1 % sodium dodecyl sulfate, 20 mM Tris buffer, pH 7.2) was added to each sample on the nuclepore filter. Following the preincubation at 30°C for 30 min, 9 μl of hybridization solution containing 2.5 μg of fluorescence isothiocyanate (FITC)-labeled probe was added. The mineral particles were hybridized at 30°C for 6 hr in a water bath, and washed with 20-40 μl of hybridization solution twice at 30°C for 20min.

After hybridization and washing, the nuclepore filter with the mineral particles was placed on a filtering device, and rinsed with distilled water. Subsequently, the filter was observed under an epifluorescence microscope (Olympus Co., Tokyo, Japan) equipped with the dichroic mirror system for FITC (excitation wavelength, 465-495nm; dichroic mirror, 505nm). Photomicrographs were taken with color reversal 400nm film (FUJIFILM, Tokyo, Japan). After a filter transect was scanned, the bacterial particles on the filter transect were counted.

**Accession numbers**

The DDBJ accession numbers for the 16S rDNA sequences determined in this study

255 are from AB740155 to AB740159 and from AB740968 to AB740970.

256

257

258 **Results**

259

260 **Environmental factors**

261 Analysis of air-mass backward trajectories revealed that the air mass of March 27th
262 2010 was carried from the Gobi Desert area and passed over the industrial area in China
263 and across the Sea of Japan (Fig. 2). During sampling period, observed weather
264 condition at 3000 m was a clear sky and clouds coved over the ground area. The mean
265 temperature at 3000 m was -16.4 °C. According to the Wajima Meteorological
266 Observatory of the Japan Meteorological Agency, westerly winds of 18 m/s were
267 recorded at 700 hPa (about 3000 m above sea level) over the north coast of Noto
268 Peninsula at 9:00 p.m. (12:00 UTC) on March 27th, 2010.

269 When the aerosol particles in air samples collected at 3000m were observed using
270 epifluorescence microscopic observation using DAPI staining, the air samples of March
271 27th included mineral particles at concentrations of 1,050 ± 790 particles/l, and yellow
272 fluorescent particles were detected at concentrations of 1,930 ± 700 particles/l (Table 1).
273 The total density of bacterial cells associated with aerosol particles was 2,280 ± 830
274 particles/l. When particle densities at 3000m were measured using a particle counters,
275 particles between 0.3 - 0.5 μm of March 27th showed high concentrations of more than
276 45,000 particles/l and made up about 85% of the total number of particles (Table 1). In
277 addition, the air mass of March 27th included the particles between 0.3 - 2.0 μm at

concentrations ranging from 430 particles/l to 4,900 particles/l, and relatively large particles >2.0 μm at a concentration of 121 particles/l.

**NaCl amended cultures**

When the air sample collected at 3000m was inoculated in TS liquid media containing different NaCl concentrations, microbial growth in the media containing 0%, 3%, and 10% NaCl rapidly increased to an absorbance of >95 (approximately $4 \times 10^7$ cells/ml) within 5 days of incubation and fluctuated between 51 and 420 during the experimental period (Fig. 3). Cultures amended with 15% NaCl began to show minimal microbial growth from the 4th day, and the absorbance gradually increased to approximately 25 over the experimental period. These results indicated that microorganisms that were tolerant to NaCl concentrations of up to 15% maintained their viability in the air sample collected on March 27th.

Colonies on the agar plates on which the NaCl amended cultures were spread were picked up judging by colony formation and colors. Consequently, total five isolates were obtained from each NaCl amended cultures including 0%, 3%, 10%, or 15% NaCl. The full sequences of 16S rDNA (ca. 1450) of the 4 isolates belonged to the group of *B. subtilis* in *Firmicutes* and indicated high similarities at >99.9% (Table 2).

**DGGE analysis of bacterial communities**

When the bacterial species composition of the air sample was determined using PCR-DGGE analysis, the gDNA extracted directly from the air sample and from the NaCl-amended cultures showed different banding patterns, with three DGGE bands and

301 one band, respectively (Fig. 4). The DGGE bands of cultures amended with 0%, 3%,
302 10%, and 15% NaCl each showed a single dominant band (SAd-2, SAd-3, SAd-4, and
303 SAd-5) at identical positions. These single dominant bands were identical to one
304 (SDd-1) of the three bands obtained from gDNA extracted directly from the sample.
305 The 16S rDNA sequences of the dominant bands (SDd-1, SAd-2, SAd-3, SAd-4, and
306 SAd-5) yielded a single phylotype that had 100% similarity to that of *B. subtilis* (Table
307 2). This indicated that a single bacterial species was common to the cultures at all NaCl
308 concentrations. The remaining two bands (SDd-6 and SDd-7) were specific to the
309 gDNA extracted directly from the air sample. The SDd-6 sequence had 98.5% similarity
310 to that of *Rhodanobacter terrae* (Table 2). The SDd-7 sequence belonged to
311 *Bacteroidetes* and had 88.8% similarity to that of *Owenweeksia hongkongensis*,
312 suggesting that the phylotype including SDd-7 was a novel bacterial species. These
313 results indicated that members of three phylotypes were abundant in the air sample and
314 that the one phylotype dominant in the troposphere could grow by enrichment culture.
315
316 **Comparison of 16S rDNA clones**
317 16S rDNA fragments (ca. 1450bp) in the air sample were amplified by PCR with
318 primers targeting eubacterial 16S rDNA. The PCR amplicons were cloned into *E. coli*,
319 and a total of 65 clones including eubacterial 16S rDNA fragments were obtained from
320 the air sample. Sequences of the 16S rDNA clones showed that the bacterial populations
321 were divided into 3 phylotypes defined as sequences with >98% sequence similarity
322 (Table 2). The majority of phylotypes were affiliated with *Firmicutes, Bacteroidetes,*
323 and *Gammaproteobacteria* lineages that are typically well represented in 16S rDNA

clone libraries generated from terrestrial and marine environments (Table 2). In particular, sequences belonging to *Firmicutes* accounted for 85% of total clones. All *Firmicutes* sequences fell into a single phylotype that was closely related to *B. subtilis* with high similarities of >99.7% and was identical to the sequence of the dominant DGGE bands (SDd-1, SAd-2, SAd-3, SAd-4, and SAd-5) and the sequence of isolates obtained from NaCl amended cultures (Fig. 5). Another phylotype, including four clones belonging to *Bacteroidetes*, was related to *O. hongkongenesis* at a low similarity between 88.3% and 88.4%, and was >98.6% identical to the sequence of DGGE band (SDd-7). The one remaining clone belonged to *Xanthomonadaceae* in *Proteobacteria* and was closely related to *Pseudoxanthomonas byssovorax* with a similarity of 93.6%.

**Whole-mineral particle *in situ* hybridization targeting bacterial cells**

Epifluorescence microscopy after whole-particles *in situ* hybridization of mineral particles collected at 3000m revealed that the probes PB-BS 28 for *B. subtilis* and Eub338R for eubacteria bound to the small particles on the surfaces of mineral particles (Fig. 6). Particles bounded with the probe non-Eub (negative control) were not observed (data not shown). The signals by PB-BS 28 occupied approximately 80% of small particles among the all small particles detected by Eub 338 for eubacteria (Table 4), suggesting that the nucleotides originated from *B. subtilis* cells dominated on the mineral particles.

**Discussion**

347 The westerly wind at high altitudes over Asian region is known to carry Kosa
348 mineral particles associated with microorganisms across hundreds and thousands of
349 kilometers, and these airborne microorganisms are dispersed around the Asian
350 downwind areas through the free troposphere (Griffin et al. 2003; Iwasaka et al. 2009).
351 The air mass over Noto Peninsula on March 27th, 2010, was carried from the
352 continental desert areas (Fig. 2) and had high amounts of aerosols and included
353 significant amount of mineral, yellow and bacterial particles (Table 1). DAPI
354 yellow-fluorescing particles have been reported to resemble organic materials
355 originating from microbial cell components such as proteins (Mostajir et al. 1995). The
356 Japan Meteorological Agency reported westerly winds of 18 m/s at 3000 m above the
357 ground during the sampling period. Furthermore, SYNOP (surface synoptic
358 observations) database indicated that dust events occurred at several sites in the
359 continental desert areas for 3 days before the sampling dates. During the spring and
360 summer seasons, the prevailing westerly winds is thought to constantly carry dust
361 particles throughout the free troposphere and cause the weak Kosa at a height of 4000 m
362 over East Asia (Iwasaka et al. 1988; Matsuki et al. 2003). Kosa events have been
363 reported to increase the number of airborne microorganisms on ground surfaces in
364 correspondence with the amount of mineral particles (Hara and Zhang 2012). During
365 the March 27th sampling period, the prevailing westerly wind is believed to carry
366 aerosol particles from continental areas to high altitudes above Noto Peninsula.
367 The NaCl amendment culture demonstrated that the air sample collected at 3000m
368 on March 27th showed significant microbial growth in the culture media including up to
369 15% NaCl (Fig. 3). Halotolerant bacteria are known to survive in extreme environments

370 through resistance to several stressors, such as desiccation, UV irradiation, extreme
371 temperatures, oxygen limitation, and high salinity (Russell 1989). Halotolerant bacteria
372 have been isolated from the ice cores of Greenland, suggesting the long-range transport
373 of the bacteria by dust events (Yukimura et al. 2009). The bacterial communities in the
374 NaCl amended cultures and the halotolerant isolates obtained from the cultures were
375 mainly composed of *B. subtilis* (Table 2). *Bacillus* spp. are known to form endospores
376 that are resistant to environmental stressors and that enhance their survival in the
377 atmosphere (Nicholson et al. 2000). Presumably, *B. subtilis* can resistant to high salinity
378 maintained its viability in the free troposphere during the sampling period, when the
379 weak Kosa is thought to occur. Halotolerant bacteria that are resistant to atmospheric
380 stressors would maintain their viabilities and be selected among entire airborne bacteria,
381 originated from ground area.

382 The sequences of *B. subtilis* growing in the NaCl-amended cultures were identical
383 to a sequence detected from gDNA collected from the March 27th air sample and were
384 abundant in the 16S rDNA clone library obtained from the sample (Table 2, Fig. 5).
385 FISH technique revealed that *B. subtilis* cells occupied approximately 80 % of total
386 cells of activated microorganisms (Table 3). The species composition of cultured
387 isolates obtained from natural environments are often different from the diverse
388 bacterial lineages detected using culture-independent techniques (Maron et al. 2005),
389 because 99% of environmental bacteria can not be cultivated by traditional methods
390 (Olsen and Bakken 1987). In contrast, the clone libraries obtained from Antarctic and
391 Arctic pack ice samples revealed strong phylotype overlap with cultivated isolates
392 (Brinkmeyer et al. 2003). In the air sample collected at 3000 m on March 27th, *B.*

393 *subtilis* would be a dominant species, and the viability of *B. subtilis* could be amended
394 using culture techniques.

395 Although this sampling was performed during a single period, two air samples
396 commonly included *B. subtilis*, indicating the high possibility that this species was
397 transported by the westerly wind. The members of *B. subtilis* group including
398 halotolerant bacteria were dominantly associated with dust mineral particles collected at
399 altitudes some hundreds of meters above the Taklamakan Desert (Maki et al. 2008) and
400 Suzu City during Kosa events (Maki et al. 2010). In the snow cover of Mt. Tateyama
401 accumulating aerosols with snow fall during the winter and spring seasons, the snow
402 layer that included Kosa mineral particles contained halotolerant bacteria identified as
403 the *B. subtilis* group but layers without dust particles did not contain *B. subtilis* (Maki et
404 al. 2011). Species related to *B. subtilis* were isolated from sand samples of the Gobi
405 Desert area (Hua et al. 2007) and reported to dominate in the surface air of Saul City
406 during Kosa events (Jeon et al. 2011). Therefore, *B. subtilis* in the air sample was
407 possibly transported with dust mineral particles from the continental desert area.

408 Although members of *B. subtilis* group are most often thought to be
409 non-pathogenic and clinical contaminants, they are considered to be serious nosocomial
410 bacteria infecting injured persons (Richard et al. 1988) and immunosuppressed patients
411 (Velasco et al. 1992). In contrast, the *B. subtilis* group included antagonists, which
412 suppress the pathogenic diseases of plants (Alabouvette et al. 1996) and cultured shrimp
413 (Banerjee et al. 2007). Furthermore, some strains of *B. subtilis* have been used for the
414 production of Japanese health foods such as *natto* (Ashiuchi et al. 1998). Communities
415 of the *B. subtilis* group are reported to degrade organic matters, thus contributing to the

carbon cycle in terrestrial environments (Das and Mukherjee 2007). Therefore, the atmospheric transports of the *B. subtilis* group might have negative and positive influence on human societies and environmental ecosystems. There is a possibility that atmospheric transport of the *B. subtilis* group influences several aspects of human societies and environmental ecosystems in Asian regions.

FISH technique revealed that the approximately 20% of eubacterial cells would be composed of minor species except for *B. subtilis* (Table 4). *Bacteroidetes* sequences detected from the clone library were identical to a DGGE band of gDNA extracted directly from the air sample (Table 2). Moreover, the clone library of air sample also included members of *Proteobacteria* belonging to the *Xanthomonadaceae* group (Fig. 5). Some species belonging to *Bacteroidetes* and *Proteobacteria* are expected to be transported by the westerly wind. The eubacterial cells bounded with the probe Eub338R showed higher concentrations than the bacteria identified with DAPI did. FISH technique was reported to identify just 40%-80% of bacteria with nucleotide probes in respect of total bacteria identified with DAPI, because the hybridization probes target to only ribosomal RNA (Lew et al. 2010). In this study, the incubation with TS medium addition would induce the proliferation of bacterial cells on mineral particles. The minor bacterial species may also maintain their viabilities in atmosphere, and the bacterial concentrations estimated by FISH were overestimated.

## Conclusion

This study reported the bacterial communities in the free troposphere over Noto Peninsula (altitudes of 3000 m) when the air masses were transported from continental areas by westerly wind. Halotolerant bacteria belonging to the *B. subtilis* group would maintain their viabilities and dominated in the free troposphere over Noto Peninsula. There are possibilities that atmospheric stressors selected halotolerant bacteria among several species of airborne bacteria originated from ground area, and that the westerly wind carried *B. subtilis* through the free troposphere. In addition, there were some species of unculturable bacteria belonging to *Proteobacteria* and *Bacteroides* in the free troposphere. In future, more clone libraries of microbial communities obtained by several sampling at high are required to determine the origin region in continental areas or sea areas. Moreover, several bioaerosol samples can be compared for establishing a database of microbial communities transported for long distance to Japan by tropospheric winds.

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595 **Figure Legends**

596

597 Fig. 1 A route of aircraft sampling (solid line) from Suzu City (White circle; 37.5°N,
598 137.4°E) to the sea area (Black circle; 37.5°N, 136.4°E) during the sampling period
599 from 14:50 to 16:50 on March 27th, 2010.

600

601 Fig. 2 Three-day backward trajectories of aerosols that arrived Suzu City on March 27th,
602 2010.

603

604 Fig. 3 Microbial growth of bioaerosol sample collected at altitudes of 3000 m on March
605 27th, 2010, in media containing NaCl at concentrations of 0 % (square), 3 % (circle), 10
606 % (triangle) and 15 % (diamond). All experiments were performed in five test tubes.

607

608 Fig. 4 DGGE profile (band patterns) of amplified 16S rDNA from genomic DNA
609 directly extracted from the air sample collected at 3000 m on March 27th, 2010, and
610 from the bacterial cultures of air sample collected at 3000 m, which were cultivated in
611 TS media containing 0 %, 3 %, 10 %, and 15 % NaCl. A 40 % (upper side) to 60 %
612 (lower side) denaturing gradient was used.

613

614 Fig. 5 Phylogenetic tree including the partial sequences of 16S rDNA amplicons
615 obtained from NaCl amended bacterial isolates, DGGE bands and 16S rDNA clones.
616 The tree was calculated from a dissimilarity matrix of a ca. 553-bp alignment (*E. coli*
617 numbering 372 to 900) using a neighbor-joining algorithm. Sequences of Szi series

618 were obtained from the bacterial isolates from the NaCl amended cultures. Sequences of
619 SzDd and SzAd series were obtained from the DGGE bands of the NaCl-amended
620 cultures and gDNA extracted directly from the air sample, respectively. SzDc-March
621 series indicate sequences of the 16S rDNA clone library. The sample information and
622 the accession number of each reference sequence are given in parentheses. Bootstrap
623 values >50% (after 1,000 resamplings) are indicated on the branches.
624
625 Fig. 6 Photographs indicating whole-mineral particles *in situ* hybridization against
626 bacterial particles attached on Kosa-mineral particles that are collected at 3000 m on
627 March 27th, 2010. Kosa-mineral particles were hybridized and stained with three
628 FITC-labeled probes, Eub338R (probe for eubacteria; a), and PB-BS 28 (probe for *B.*
629 *subtilis*; b). Arrows in the micrographs show outline of the bacterial cells detected by
630 FITC-labeled probes. All photomicrographs were taken at a magnification of x1000.
631 (scale bar shows 5μm).

Figure
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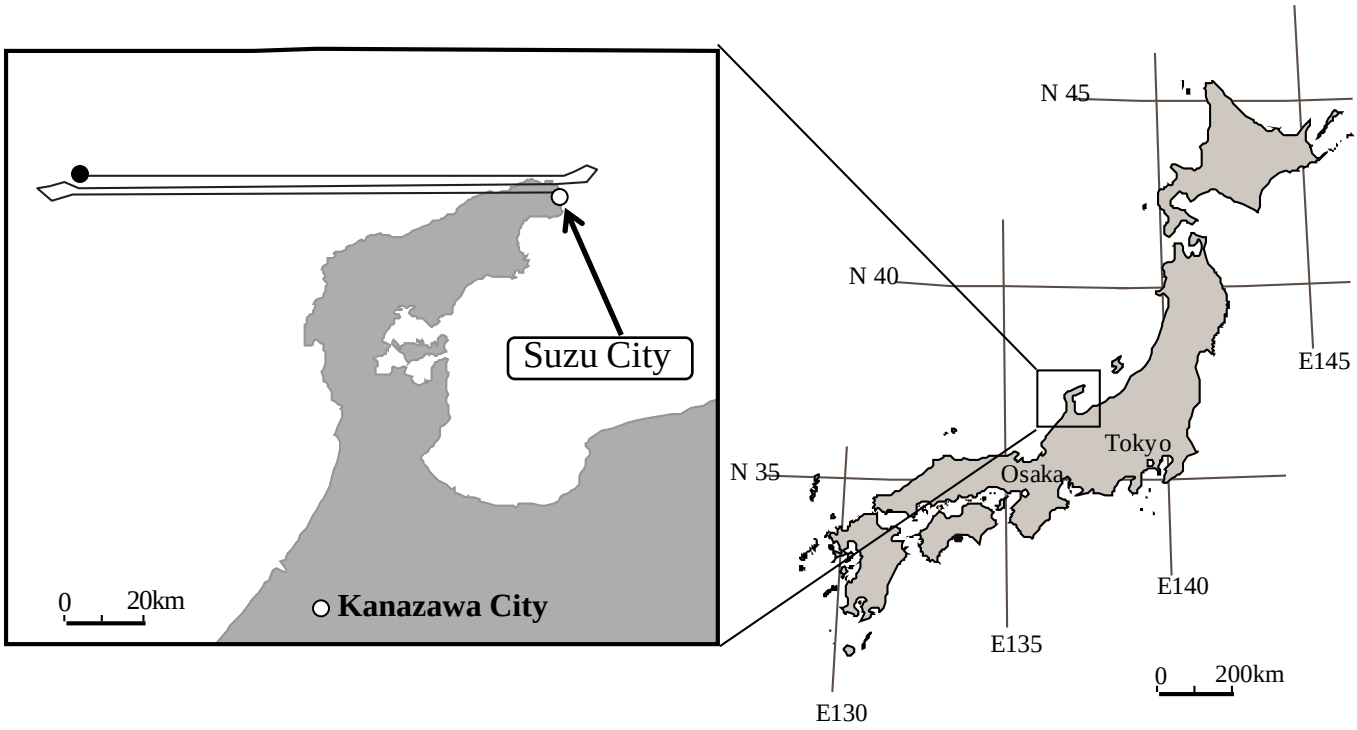
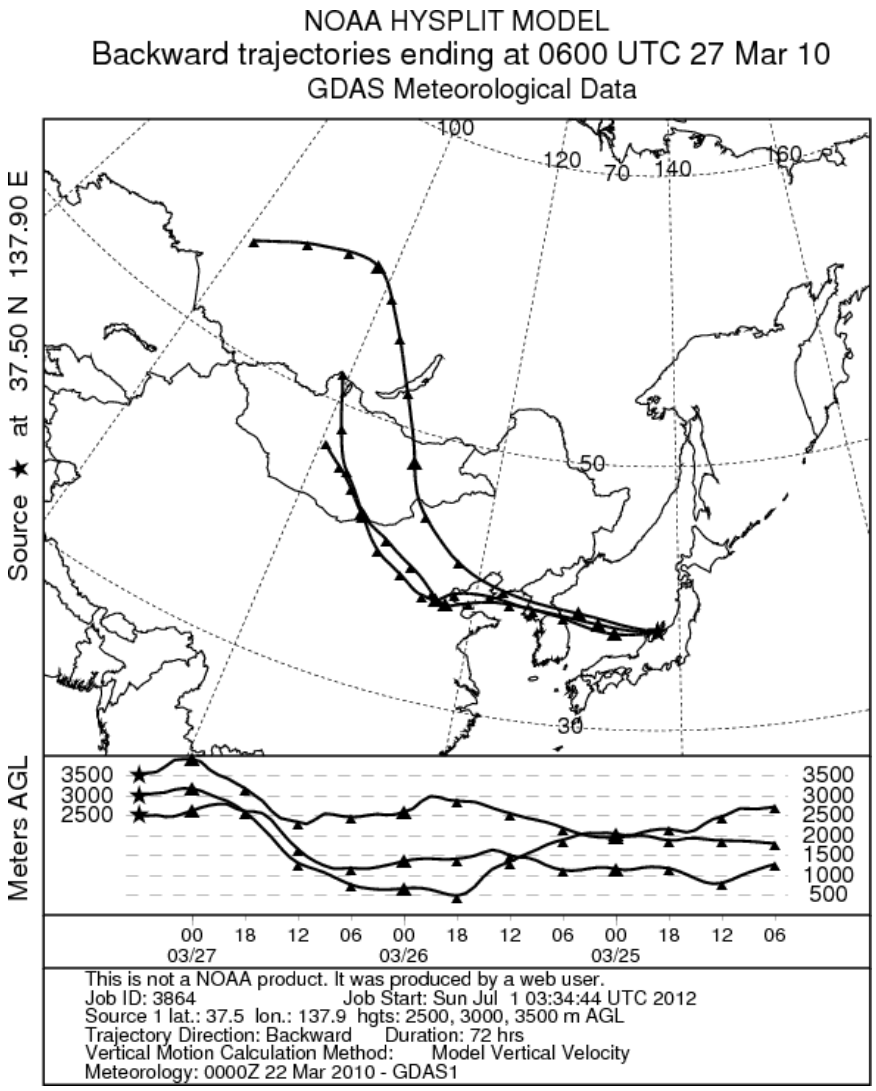



Fig. 1 T.Maki et al.

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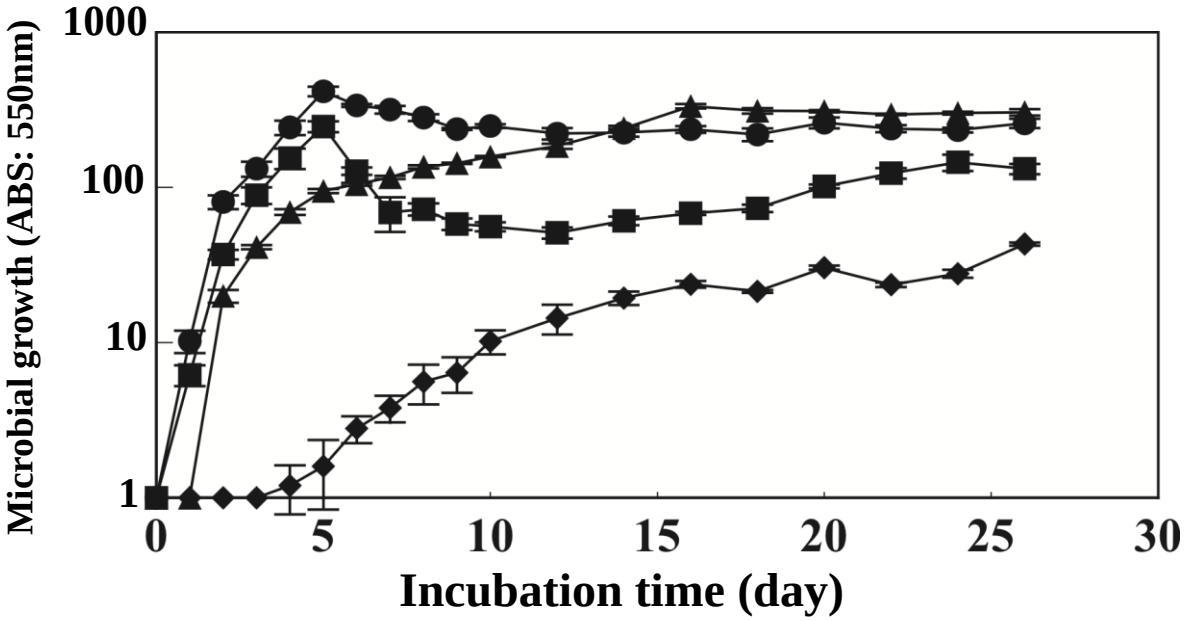


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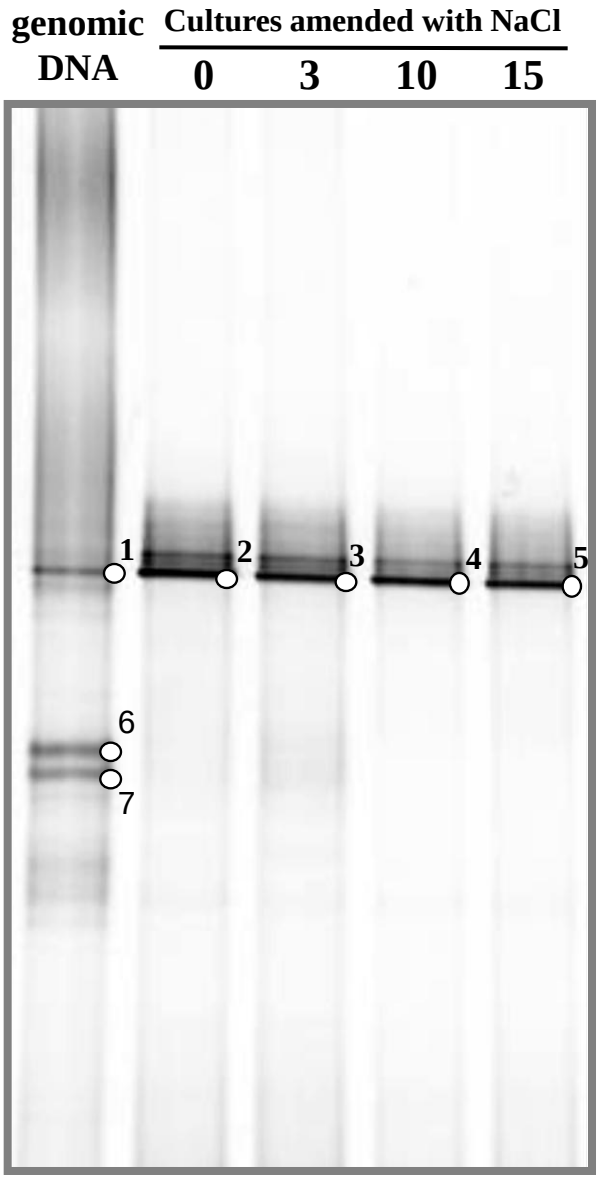


Fig. 4 T.Maki et al.

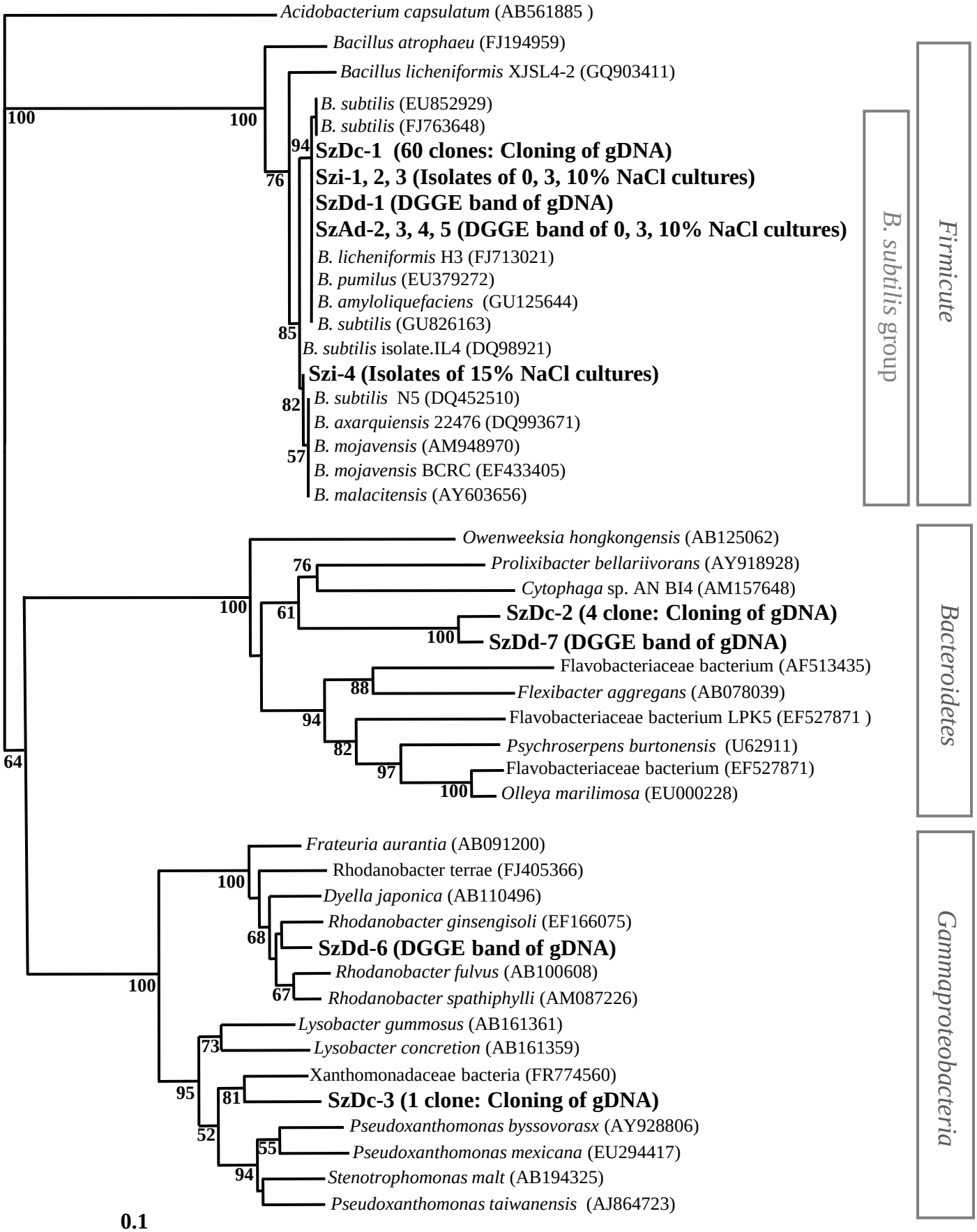


Fig. 5 T. Maki et al.

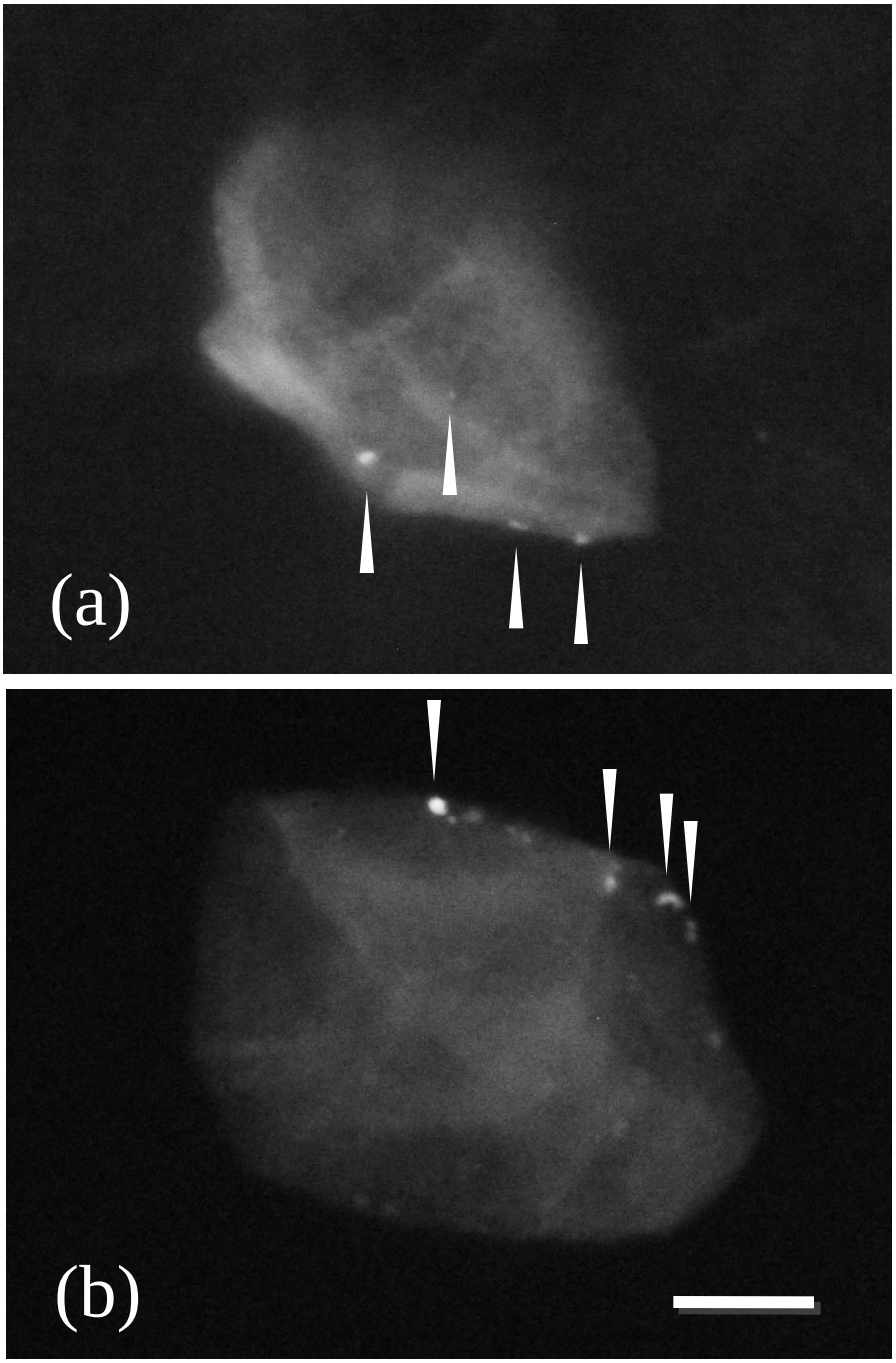


Fig. 6 T. Maki et al.

Table
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**Table 1. Concentrations of particles in the air-samples collected at 3000m on March 27th, 2010.**

| Analytical methods | Categories of partilces | Particle concentrations in air mass (particles/l) |
|---|---|---|
| Microscopic observation | Mineral particles | 1050 ± 790 |
| | Yellow particles | 1930 ± 700 |
| | Bacterial cells | 2280 ± 830 |
| Particle counter | 0.3 - <0.5* | 46600 ± 8400 |
| | 0.5 - <0.7 | 4900 ± 1100 |
| | 0.7 - <1.0 | 1020 ± 180 |
| | 1.0 - <2.0 | 433 ± 78 |
| | 2.0 - <5.0 | 117 ± 33 |
| | >5.0 (µm) | 3.97 ± 2.38 |

**Table 2. Phylogenetic affiliation of sequences of bacterial isolates, DGGE bands, and 16S rDNAclones obtained from the air-sample.**

| Analytical methods | Names of sequences*1 | Numbers of sequences*2 | Conditon*3 | Length (bp) | Category | GenBank accession no. | Closest relative | Similarity (%)*4 |
|---|---|---|---|---|---|---|---|---|
| Isolates | Szi-1, 2, 3 | 3 | <10%NaCl | 1409 | *Firmicutes* | AB740155 | *Bacillus subtilis* (GU826163) | 100 |
| | Szi-4 | 1 | 15%NaCl | 1426 | *Firmicutes* | AB740156 | *Bacillus subtilis* (HQ425655) | 99.9 |
| PCR-DGGE analysis | SzDd-1<br>SzAd-2, 3, 4, 5 | 6 | <15%NaCl<br>directly extracted DNA | 542 | *Firmicutes* | AB740968 | *Bacillus subtilis* (GU826163) | 100 |
| | SzDd-6 | 1 | directly extracted DNA | 548 | *Gammaproteobacteri* | AB740969 | *Rhodanobacter terrae* | 98.5 |
| | SzDd-7 | 1 | directly extracted DNA | 546 | *Bacteroidetes* | AB740970 | *Owenweeksia hongkongensis* | 88.8 |
| Clone library | SzDc-1 | 60 | directly extracted DNA | 1452 | *Firmicutes* | AB740157 | *Bacillus subtilis* (GU826163) | 99.8 |
| | SzDc-2 | 4 | directly extracted DNA | 1394 | *Bacteroidetes* | AB740158 | *Owenweeksia hongkongensis* | 88.3-88.4 |
| | SzDc-3 | 1 | directly extracted DNA | 1349 | *Gammaproteobacteric* | AB740159 | *Pseudoxanthomonas byssovorax* | 93.6 |

***1 Isolates from the NaCl amended cultures are named as the Szi serie . DGGE bands in Fig. 4 refer to the SzAd or SzDd series. Clones of 16S rDNA library were named as the SzDc.**

***2 Numbers of NaCl amended bacterial isolates, DGGE bands, and 16S rDNA clones.**

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

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

**Table 3. Consentrations of bacterial particles in the sir-sample, which were detected by FISH technique.**

| Targets of nucreotide robes | Concentrations of particles in air mass (particles/l) | Rates of large particles with bacterial aggregates (%) | Particle numbers on each large particles (particles) | Rates of FISH stained particles to DAPI stained particles (%) |
|---|---|---|---|---|
| ***B. subtilis*** | **$2.49 \times 10^3$** | **13.8** | **4.5 ± 1.7** | **77.5 ± 14.7** |
| **Eubacteria** | **$2.99 \times 10^3$** | **14.4** | **5.2 ± 1.4** | **94.9 ± 10.8** |
| **negative control** | **N.D*** | **N.D*** | **N.D*** | **N.D*** |

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

attachment to manuscript

# Dust events (present weather) recorded by SYNOP

Click here to download attachment to manuscript: Attachment_Map_dust synop distribution.ppt

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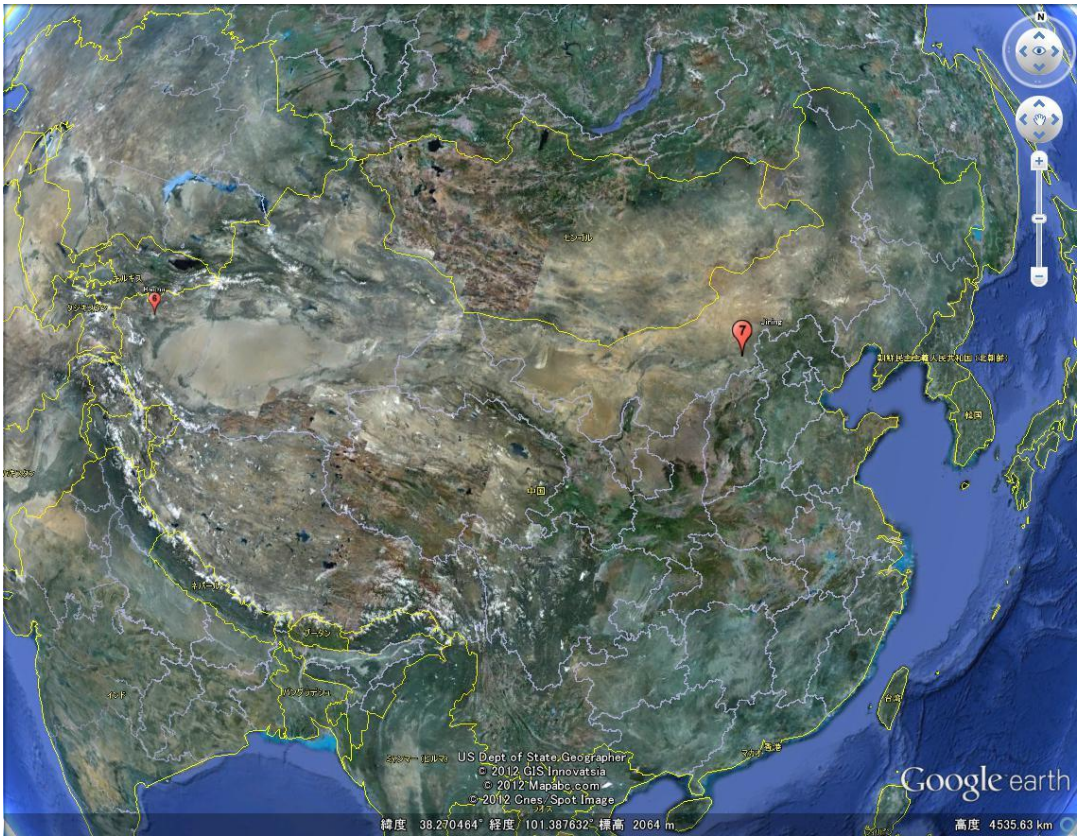
0000-2100UTC,
Mar. 25, 10’

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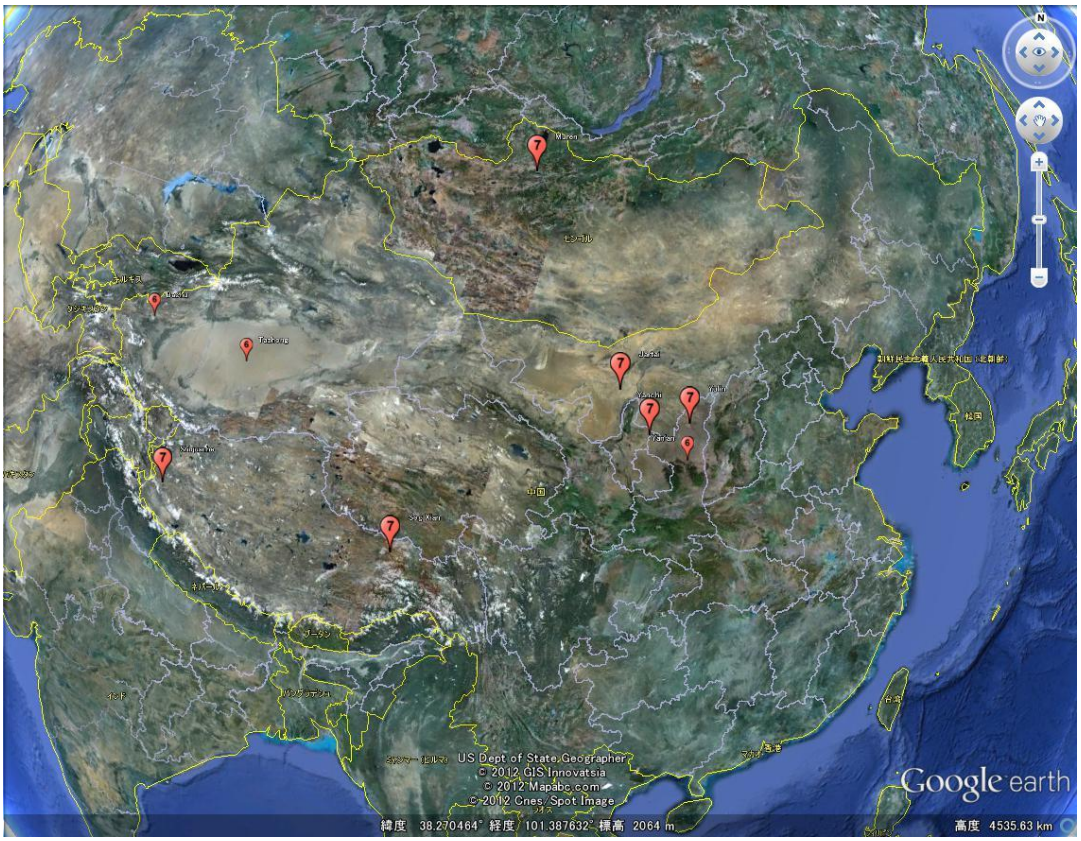

Dust events (present weather) recorded by SYNOP

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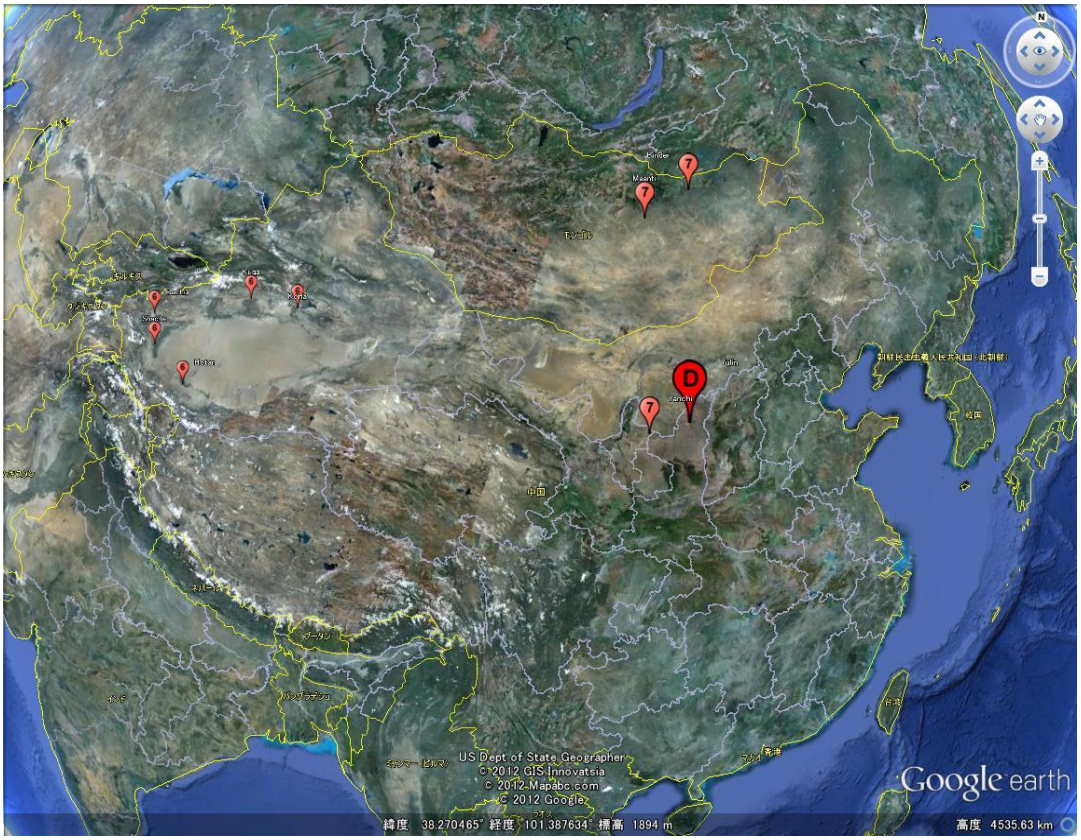


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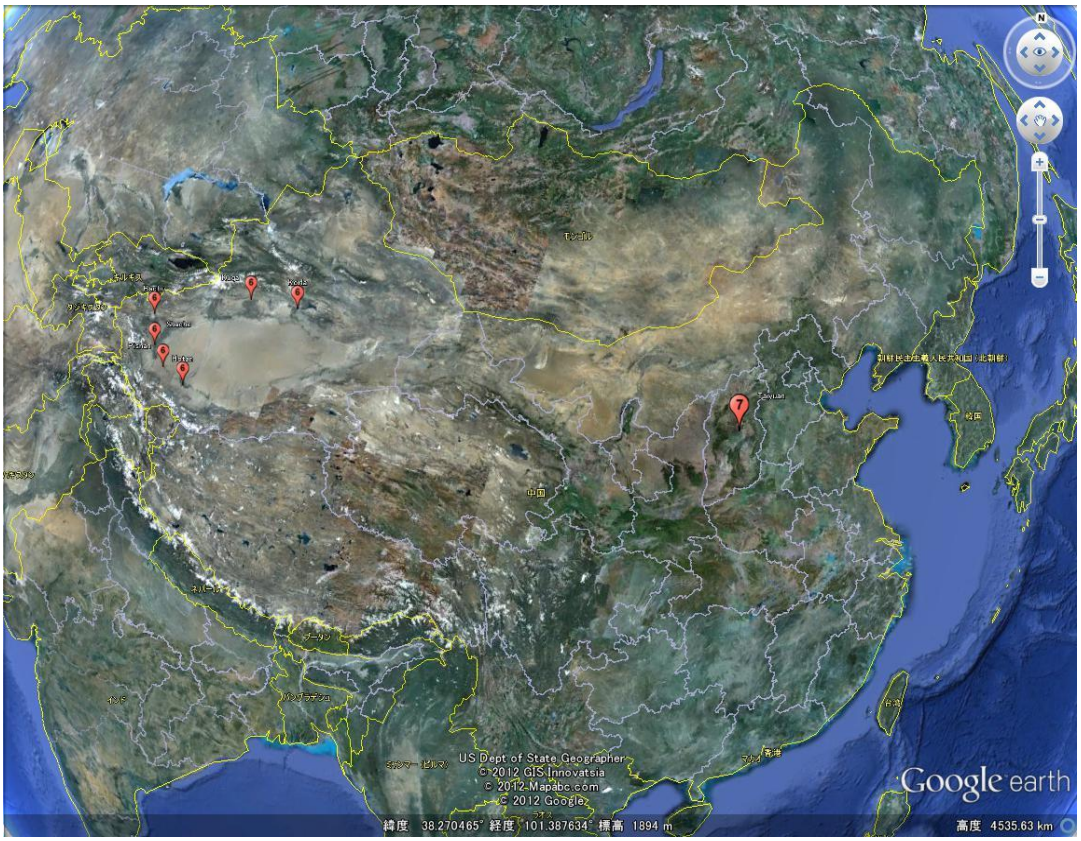
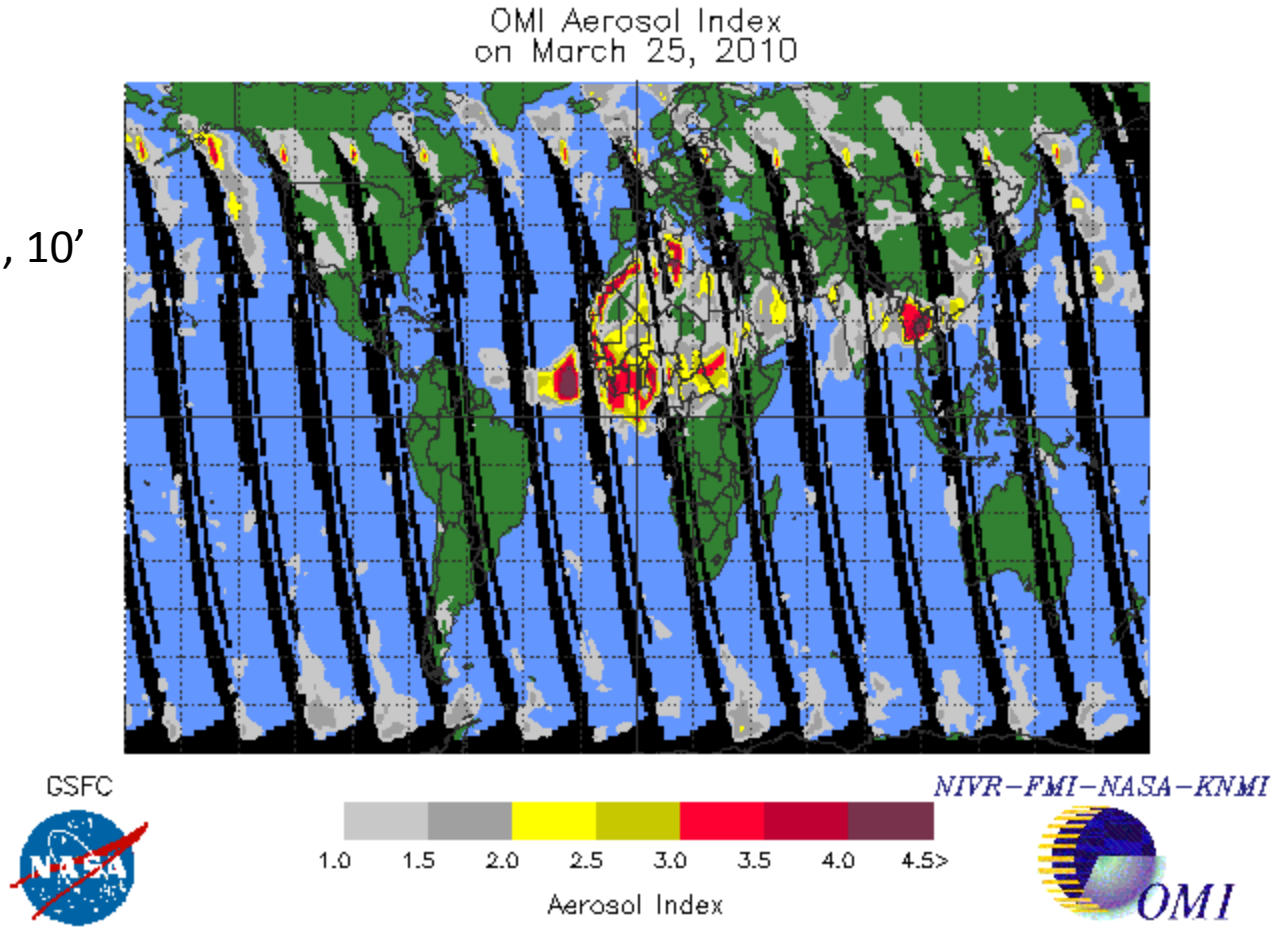


OMI Aerosol Index

Mar. 25, 10’

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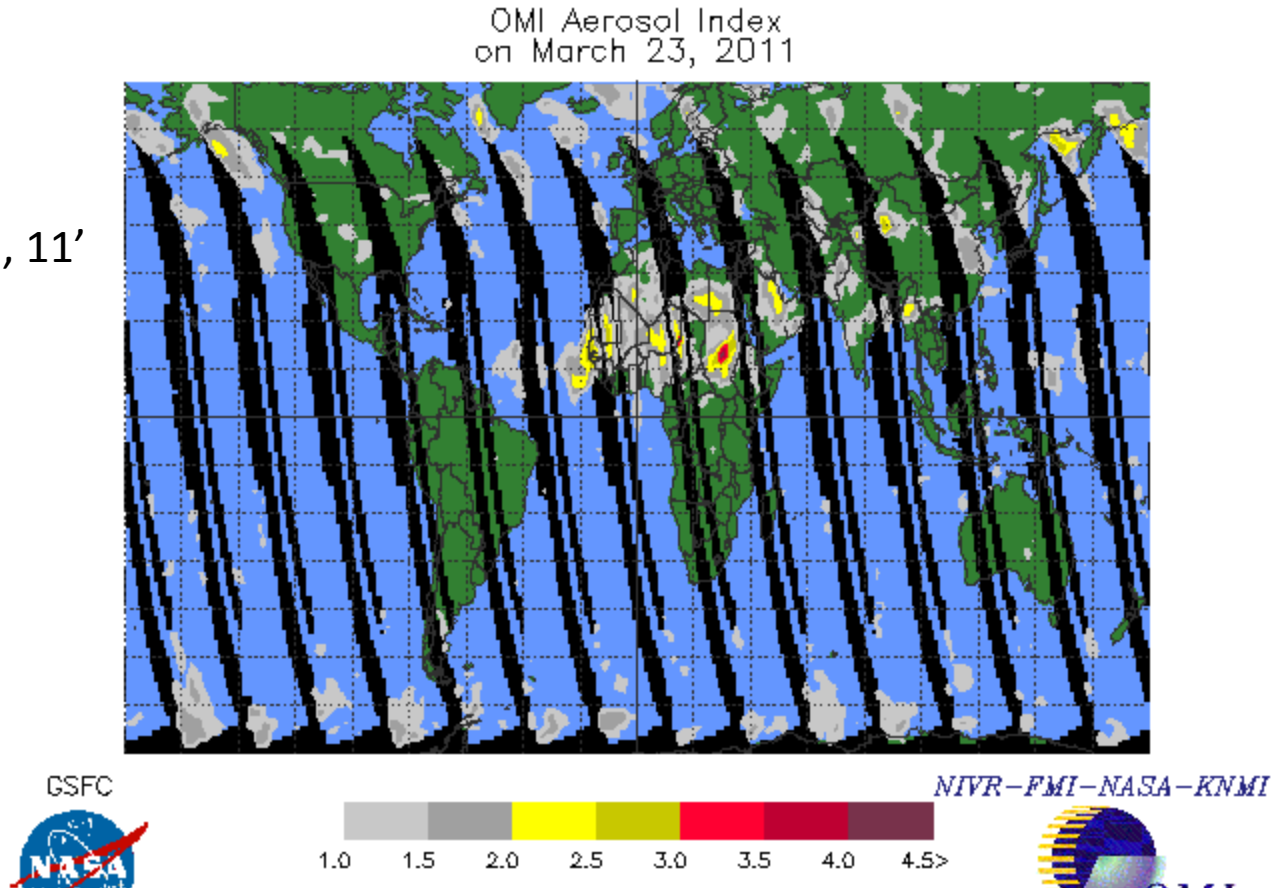
Mar. 23, 11’

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