Running head: **Dust-borne bacteria from Gobi Desert**

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Title: **Detailed identification of desert-originating bacteria carried by Asian dust storms to Japan**

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Abstract

Several halotolerant bacteria were isolated from dust settled passively in saline medium (supplemented with 150 g L^{-1}) in Higashi-hiroshima, Japan during the Asia dust events in 2005-2006. The primary identification based on 16S rRNA gene revealed that these isolates were strains of *Bacillus subtilis*, *Bacillus licheniformis*, *Staphylococcus epidermidis*, *Gracillibacillus* sp., and *Halomonas venusta*. A parallel investigation was done on desert sand collected directly in Dunhuang, Gobi region, China that resulted in revivification of seven bacterial strains which were highly identical to *B. subtilis* strains and the *B. licheniformis* strain obtained previously (99.7% and 100% of 16S rDNA sequence similarity, respectively). A further genetic analysis based on the universally house-keeping genes, *gyrB* and *parE* was done on the group of *B. licheniformis*. The data indicated high sequence similarities in both genes among the strains of both locations (99.0–99.4%) that clustered them in a monophyletic line. Phenotype characterized by numerical taxonomy for 150 physiological tests again confirmed the extreme relatedness between strains (similarity coefficient, S_{SM} = 96.0%). The excellent agreement of phenotype and genotype of the bacterial isolates lead to a conclusion that there was an aerosolized dispersion of a *B. licheniformis* population living in Gobi Desert to nearby regions by dust storms. The present paper shows a direct evidence of microbial transportation caused by yellow dust event in North-East Asia.

Keywords: Bacillus licheniformis, dust-borne bacteria, Gobi Desert, halotolerant, numerical taxonomy, yellow dust

1 Introduction

The intercontinental transportation of million tons of desert dust annually plays an important role to the whole ecosystem on Earth (Duce et al., 1980; Parrington et al., 1983;

Betzer et al., 1988; Uematsu et al., 2002; Jickells et al., 2005). While the inorganic fertilization consequently derived from this natural phenomenon may affect beneficially downwind ecosystems the associated pollutants and pathogenic microbes cause adverse effects to animals, plants, and human lives and makes environments become worst (Schlesinger et al., 1990; Young et al., 1991; Uematsu et al., 1992; Griffin et al., 2002; Kwon et al., 2002; Griffin and Kellogg, 2004). Air-borne miroorganisms found so far represented diverse groups, some were as common inhabitants of soil, terrestrial aquatic, and marine environments; others were present in association with desert dust storms; and few others were found present in aerosol at an high altitude of 20,000 m (Bauer et al., 2002; Griffin et al., 2003; Griffin, 2004; Echigo et al., 2005; Prospero et al., 2005; Griffin et al., 2006; Shivaji et al., 2006; Brodie et al., 2007). Among of these groups, desert dust borne microbes would be the most effective to the downwind ecosystem because they are extremely resistant to harsh conditions and hard survivors from long journey dispersion. In addition, dormant spores originated from deserts may possess unknown properties related to ancient age of these habitats. There have been several researches determining dust borne microorganims in African desert system and the ecological consequences to the nearby regions (Shinn et al., 2000; Griffin et al., 2003, 2006; Kellogg et al., 2004; Prospero et al., 2005). In Asia, the Yellow Dust associated microbial pollution derived from the world's second largest desert system located in China and Mongolia was just reported recently (Yongyi et al., 1993; Choi et al., 1997; Ho et al., 2005). Prior to numeric determinations of fungal spores in yellow dust-polluted air in Taiwan and South Korea (Yeo and Kim, 2002; Wu et al., 2004), Kwon et al. (2002) showed that community health in South Korea was affected seriously by dust events and the public death rate increased 1.7% in general. Next to Korea, Japan also suffers much yellow dust storms from China every Spring times. Echigo et al. (2005) successfully isolated and identified many halophilic bacteria from soil surface of non-saline habitats around Tokyo and argued that endospores of these bacteria would had been distributed evenly by Asian yellow storms. Although a significant number of taxa of dust borne micororganisms has been isolated and characterized in both African and Asian desert systems, many studies were done

based on culture-based enumeration and microscopic observation that resulted in limited identification at genus level and therefore led to difficulty in concluding which microorganisms associated with dust and which present in local air. The aim of this study was to isolate extremely tolerant bacteria survived from Asian yellow dust storm and to determine exactly original habitats of the isolates. The excellent match in phenotypic and genotypic characteristics of bacterial isolates obtained in Higashihiroshima, Japan and in Dunhuang, China is a very rare case that provides a direct evidence of bacterial high tolerance to long journey transportation and the biogeographical existence of *Bacillus* populations.

2 Materials and Methods

2.1 Sampling and bacterial culture

A strain of *Bacillus licheniformis*, strain L1 was isolated from a sand dune sample collected in Tottori prefecture, Japan in 2004. "Yellow sand" was collected directly in Dunhuang (40º10'00"N, 94º40'60"E), China in 2006.

Yellow dust storm happens during March to April every year starting from China and reaching Japan 3 days later. Dust samplings were followed forecasts of Japan Meteorological Agency (map illustrated in Figure 1). A passive sampling method was applied to collect dust using saline medium made of Marine Broth (Difo, MD) plus 15% NaCl (150 g L^{-1}). Agar and liquid media were prepared aseptically in Petri-disks, in 500 ml-beakers and 50-ml tubes (n=10 each, 4 times), kept open randomly on the top of a building of Hiroshima University (Higashihiroshima, 34º25'25"N, 132º44'46"E) at about 25 m high. During the yellow storm event, dust heavily covered sky of Japan and settled in the opened media for 24-36 h. Incubation was then carried out at 37ºC for 1-2 weeks. The cultures were streaked on agar medium and single different colonies were picked to produce pure cultures after at least 3 generations. Totally 9 halotolerant bacteria were obtained from 4 dust samples (DstI-DstIV) collected from February 2005 to April 2006. (Table 1). Media used for all physiological tests were made of Bacto peptone (5 g L^{-1}), Bacto meat extract (3 g L^{-1}) (Becton, Dickinson and Co.,

MD), and 0.1 g L^{-1} $MnSO_4$ x H_2O (DSMZ medium 1) plus NaCl at varied amounts (0-200 g L^{-1}) in distilled water, pH 7.0. A standard strain, *Bacillus licheniformis* DSM 603 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and used as the closest reference.

2.2 Physiological characterization

To determine phenotypic identity of strains the *B. licheniformis* group, detailed numerical taxonomy was done on isolates DstI-4, YS2-1, L1, and strain DSM 603. They were tested at different temperatures of range 10-60ºC (5ºC interval), salt concentrations of 0-20% (2% interval) NaCl at 37ºC. Oxidase and urease activity and starch hydrolysis examinations were followed methods described by Smibert and Krieg (1981). Single carbon utilization of 95 substrates was tested on GP2 microplates (Biolog) (Garland and Mills, 1991). Acid production and hydrolysis of polymers were determined using 50-well strips, API 50CH kits (Biomerieux). These tests were done triplicates at 0% NaCl, results within 24 h to 1 week were recorded and numerically coded to analyze with SPSS software (SPSS Inc.), applying simple coefficient (S_{SM}) and Jaccard coefficient (S_J).

2.3 Genetic analysis

Genomic DNA extraction was followed conventional method (Wilson, 1995). Primers and polymer chain reaction (PCR) procedures to amplify 16S rRNA gene were reported previously (DeLong, 1992). The universally single-copy conserved *gyrB* gene encoding DNA gyrase *ß* subunit and *parE* encoding DNA topoisomerase IV subunit B were amplified from genomic DNA with newly designed primers YF3 (5'-TAT AAR GTN TCN GGH GGN YTR CAC-3') (nucleotide 325-348, *E. coli* K-12 numbering) and YR3 (5'-YTT NGC NGA NCC NCC NGC NGA RTC-3') (nucleotide 1299-1264 *E. coli* K-12 numbering). Thermal profile to amplify these genes was 30 cycles of 94ºC-denaturation in 30 sec, 57ºC-annealin in 40 sec, and 72ºC-elongation in 1 min after a brief denaturation at 95ºC for 3 min and followed an elongation at 72ºC in 10 min. PCR products were cloned using TOPO TA Cloning Kit (Invitrogen, CA). The

amplified 16S rDNA (~1400 bp) and clones of *gyrB* and *parE* fragments (~1000 bp and 1220 bp, respectively) were sequenced by service of Macrogen Inc. (http://www.macrogen.com). Sequences of genes were identified and analysed with tools of EMBL (http://www.ebi.ac.uk/embl/). Phylogenetic analysis was done with MEGA 3.1 (Kumar *et al.*, 2004). DNA sequences were deposited in GenBank/EMBL/DDBJ under accession numbers of AB305265-AB305277 and AB307800-AB307807.

3 Results and Discussions

3.1 Yellow dust-borne bacteria

Totally 9 strains were obtained from 4 samples collected in 2005-2006. The bacteria were all extremely halotolerant (Table 1) which are able to grow either without or with salt of up to 16%, higher than 2.5 M (Kushner, 1978). Of them, five strains were endospore-formers belonged to family *Bacillaceae* (strains DstI-2, DstI-4, DstIII-1, DstIII-2, and DstIV-1), two strains (DstI-1 and DstI-3) were relatives of the widely-distributing bacterium, *Staphylococcus epidermidis* (100%) and 2 others (strains DstII-1 and DstII-2) were strains of marine-originated species, *Halomonas venusta* type strain DSM 4743 (99.7%). With a medium containing 15% NaCl used in screening halophiles, commonly few bacterial isolates were able to grow. The small number of isolates obtained in this study would also reflect low survival rate caused by atmospheric stresses such as UV light, toxic gases and acids during the transportation (Griffin, 2005). Echigo et al. (2005) also calculated a ratio of endospores of halophilic bacteria that assumed to be distributed by yellow storm to total numbers of bacterial endospores in terrestrial surface as small as 1/20,000. Bacteria obtained in different batches of dust were not in the same taxa. Source of microbes carried by wind may depend on extent and direction of the storm, or topology of the distance. Halomonads found in the dust would had been swept up from salt lakes in China and South Korea, or sea fog nearby. *Staphylococcus epidermidis* is a well-known human pathogen that exists widely in the atmosphere according to many reports for decades (Yongyi et al., 1993; Rupp and Archer, 1994; Kumer et al., 1996; Montacutelli et al., 2000; Zhang et al., 2003).

3.2 Bacteria isolated from Gobi Desert

Dunhuang is known as an oasis located in Gobi region, famous with sand dunes as high as mountains. Dry sand collected there was subjected to the same treatment done with dust and seven extremely halotolerant isolates were revived. They were identifies as individuals of two populations, six strains (YS1-5 and YS2-1~5) were close relatives of *Bacillus licheniformis* and one (strain YS1-4) was a member of *Bacillus subtilis* (Table 1). Within each group, these isolates were almost identical in 16S rDNA sequence (99-100%). Interestingly, they also matched 99.7-100% with strains isolated from yellow dust obtained previously. Although no strain of *Gracilibacillus* spp. was found in these samples like those in the wind-borne dust (strains DstIII-1, DstIII-2 and DstIV-1), homology search in the databases showed that closest relatives (98%) of them have been reported to be from salt lakes in Inner Mongolia, China (Table 1).

3.3 Genotypic and phenotypic evidence for desert origin of the dust borne *Bacillus licheniformis* strains

The six strains of *Bacillus licheniformis* isolated from desert sand were 99.9-100% identical to the dust-derived strain DstI-4 and strain DSM 603 in 16S rDNA sequence and clustered into a monophyletic line in any phylogenetic tree (Figure 2). To solve this ambiguity in differentiating them based on 16S rRNA gene, more variable universal protein coding genes, *gyrB* and *parE* were investigated. Sequence similarity of *gyrB* gene has been used conventionally in bacterial identification and classification, for example 88.3-99.1% for interspecies of genera *Salmonella*, *Shigella, Escherichia, Enterobacter,* and *Klebsiella* in family *Enterobacteriaceae* (Fukushima et al., 2002). Similarly, within the genus *Bacillus*, *gyrB* gene indicates 68.9-77.9% similarity between species and 99.1-100% is the range for intra-species level (our observation on full length sequences of *gyrB* gene in whole genome database, data not shown). In this study, sequences of *gyrB* and *parE* genes of three strains DSM 603, DstI-4, and YS2-1 matched 99.2%-99.4% with those of one another and very

183 divergent to those of strain L1 (about 95% and 60%, respectively). A phylogenetic tree based
184 on combined sequence sets of *gyrB* and *parE* genes (Figure 3a) indicated that the strains are
185 most likely identical.
186 Despite highly genotypic identity, strains from different origins would show different
187 phenotypic features. Therefore, a set of 150 physiological tests was done on the *B.*
188 *licheniformis* group. Significant differences between the four strains tested were carbon
189 utilizations and acid productions, yielding numerical taxonomic similarity coefficients (S_{SM})
190 of 96.0% between strain DstI-4 and YS2-1, 90.6%, and 87.2% between them and strains L1
191 and DSM 603, respectively. In this analysis, strain DSM 603 was far clustered from the dust-
192 related pair, DstI-4-and-YS2-1 (Figure 3b). Notable differences of these two bacteria
193 compared to other *Bacilli* and other strains of *B. licheniformis* were their growth at salinity
194 as high as 16% and at temperature of 60ºC (Palmisano et al., 2001). Their extreme tolerances
195 would have resulted from adaptation to desert harsh conditions.
196 Populations of *Bacillus* species commonly distribute in arid areas such as Sahara, Mojave,
197 Sonoran and Gobi Deserts (Duncan et al., 1994; Roberts & Cohan, 1995; Palmisano et al.,
198 2001), where *B. sonorensis*, the most closely related species to *B. licheniformis* was isolated.
199 Yongyi et al. (1993) reported that in the airborne bacterial community determined in Beijing,
200 *Bacillus* populations were most abundant together with *Staphylococci*. Many different
201 ecotypes of species *B. licheniformis* have been explored for industrial applications as well as
202 some of them are known to be toxic, for example in food poisoning (Salkinoja-Salonen et al.,
203 1999; Veith et al., 2004).
204 In the phylogenetic tree bsed on 16S rRNA gene (Fig 2), the *Grcilibacillus*-clustered strains
205 (DstIII-1, DstIII-2, and DstIV-1) were closely related to the arid Chinese strains such as
206 BH235, EJ-15, XH-63 (16S rRNA gene accession numbers of AY762980, AM040718, and
207 AM040716, respectively). The *B. subtilis*-clustered strains (DstI-2 and YS1-4) were closely
208 related to the arid Chinese strains/clone GCNB5, CICC 10034, Ni36, and B609 (DQ834373,
209 AY881640, DQ643186 and DQ290002, respectively). The *B. licheniformis*-clustered strains
210 DstI-4 and YS2-1 were almost identical to Chinese strains K19, BPCRC 15413, and CCBAU

10724 (DQ351932, DQ993676, and EF405624, respectively). These relationships also may support the idea of desert origins of the dust-borne strains.

It has been known that bacteria and fungi responsible for serious diseases in human, animals and plants were identified from airborne dust samples in Africa and Asia (Kwon et al., 2002; Kellogg et al., 2004; Kellogg and Griffin, 2006). Although in extent of this work, non-halotolerant bacteria, viruses and fungi are not focused, it is believed that Asian dust storm does bring these factors. We surely remember terribly huge losses caused by the severe acute respiratory syndrome (SARS) and viral bird flu recent years in Asia that may be still active elsewhere. The increasing frequency of dust storm in Asia is thought to attribute to less precipitation and prolonged drought that in turn promotes desertification (Quian, 2002). Consequently, climate, and yellow storm would be more severe in the coming years. To present status there would be lots of work expected to do for this regional dust-borne microbiology.

4 Conclusions

Asian dust storm has carried microorganisms over a long distance to Japan. Dust borne bacterial isolates are highly tolerant to high salt concentration and temperature. The populations of *B. subtilis* and *B. licheniformis* were found in both samples of the Gobi Desert and settled dust at genetic high identities. The *B.licheniformis* isolate obtained in Higashihiroshima (Japan) is a member of a population living in Duhuang (China) arid area based on genotypic and phenotypic analyses.

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Figure Legends

Figure 1. A meteorological map showing areas in Korea and Japan covered by a yellow dust storm started from Gobi Desert in March 31, 2007 adapted from a predicted map of Japan Meteorological Agency. Filled circle black spots indicate sites affected by yellow dust. Sampling sites, Higashihiroshima and Dunhuang, are marked.

Figure 2. Neighbor-joining tree showing phylogenetic clusters of dust-borne and desert-related isolates with other selected bacteria. Bootstrap values of 1000 replications greater than 50% are shown at nodes of tree. Bold names indicate strains obtained in this study. 16S rRNA gene sequence accession numbers are put in parentheses. Scale bar, 0.02 nucleotide substitution per site. *Bacillus licheniformis* cluster includes isolates DstI-4 (AB305269), YS1-5 (AB305271), YS2-1 (AB305272), YS2-2 (AB305273), YS2-3 (AB305274), YS2-4 (AB305275), YS2-5 (AB305276), L1 (AB305265), and reference strains DSM 603 (DQ081997), DSM 13 (NC006322), CICC 10107 (DQ112220), K19 (DQ351932), BCRC 15413 (DQ993676), CICC 10181 (AY842871), CCBAU 10724 (EF405624); *Bacillus subtilis* cluster includes isolates DstI-2 (AB305268), YS1-4 (AB305270), AU30 (EF032678), clone B609 (DQ290002), Ni36 (DQ643186), GCNB5 (DQ834373), CICC 10034 (AY881640).

Figure 3. a, UPGMA tree showing a monophyletic line of isolates DstI-4. YS2-1 and strain DSM 603 compared to other related bacteria based on composites of sequence of *gyrB* and *parE* genes. Bootstrap values of 1000 replications greater than 50% are put at nodes. Scale bar indicates nucleotide sequence similarity (%). b, an average linkage dendogram inferred from similarity coefficients (S_{SM} and S_J) of numerical taxonomic analysis based on data of 150 physiological tests. Names and cell micrographs (bar, 10 μm) of strains are noted in the end of the dendogram.

404 **Table 1.** Samples and bacterial isolates obtained in this study

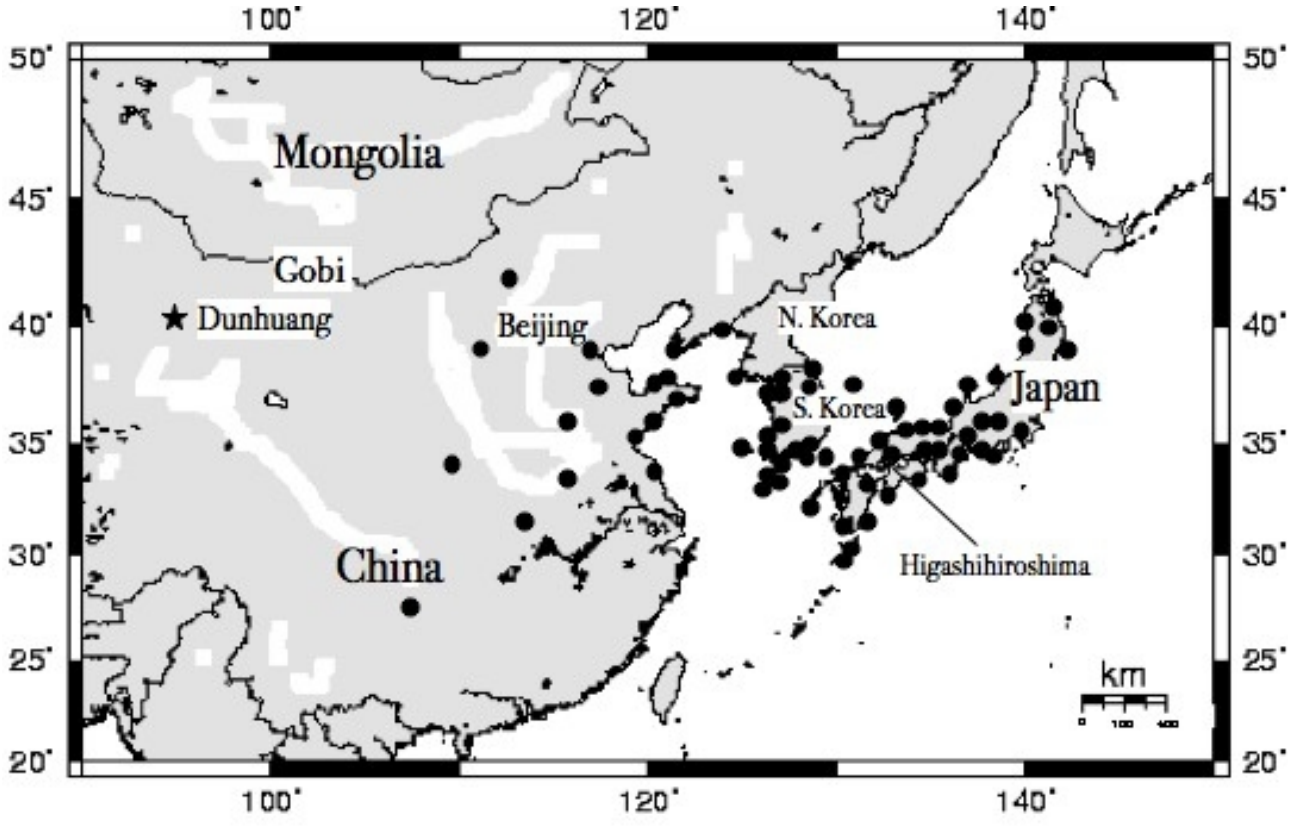
Sampling sites	Sample	Isolate	Closest bacteria		
			Species/strain[a]	16S rDNA accession No. (similarity, %)	Origin[b]
Higashihiroshima (34°25'25"N, 132°44'46"E)	DstI	DstI-1	*Staphylococcus epidermidis* (ATCC 12228)	AE015929 (100)	Human skin
		DstI-2	*Bacillus* sp. GCNB5	DQ834373 (100)	*Glycyrrhiza uralensis*, China
			Bacillus subtilis (CICC 10034)	AY881640 (100)	China
			Bacillus sp. Ni36	DQ643186 (100)	China
			Clone B609	DQ290002 (100)	China
		DstI-3	*Staphylococcus epidermidis* (ATCC 12228)	AE015929 (100)	Human skin
		DstI-4	*Bacillus licheniformis* (DSM 603)	DQ082997 (99.9)	Soil, multiple
			Bacillus licheniformis (K19)	DQ351932 (99.9)	China
			Bacillus licheniformis (BPCRC 15413)	DQ993676 (99.9)	China
				EF405624 (99.9)	China
	DstII	DstII-1	*Bacillus licheniformis* (CCBAU 10724)	AJ306894 (99.7)	Hawaii, USA
		DstII-2		AJ306894 (99.7)	Hawaii, USA
	DstIII	DstIII-1	*Halomonas venusta* (DSM 4743)	AY762980 (98.2)	China
			Halomonas venusta (DSM 4743)	AM040718 (98.0)	Saline lake, China
			Gracilibacillus sp. BH235	AM040716 (98.0)	Saline lake, China
		DstIII-2	*Gracilibacillus* sp. EJ-15		
	DstIV	DstIV-1	*Gracilibacillus* sp. XH-63		
			Like those for DstIII-1		
			Like those for DstIII-1		
Dunhuang	YS1	YS1-4	Like those for DstI-2		

(40º10’00”N,		YS1-5	Like those for DstI-4
94º40’60”E)		YS2-1	Like those for DstI-4
	YS2	YS2-2	Like those for DstI-4
		YS2-3	Like those for DstI-4
		YS2-4	Like those for DstI-4
		YS2-5	Like those for DstI-4

405 [a] more than one closest species/strains which firstly found in China are listed

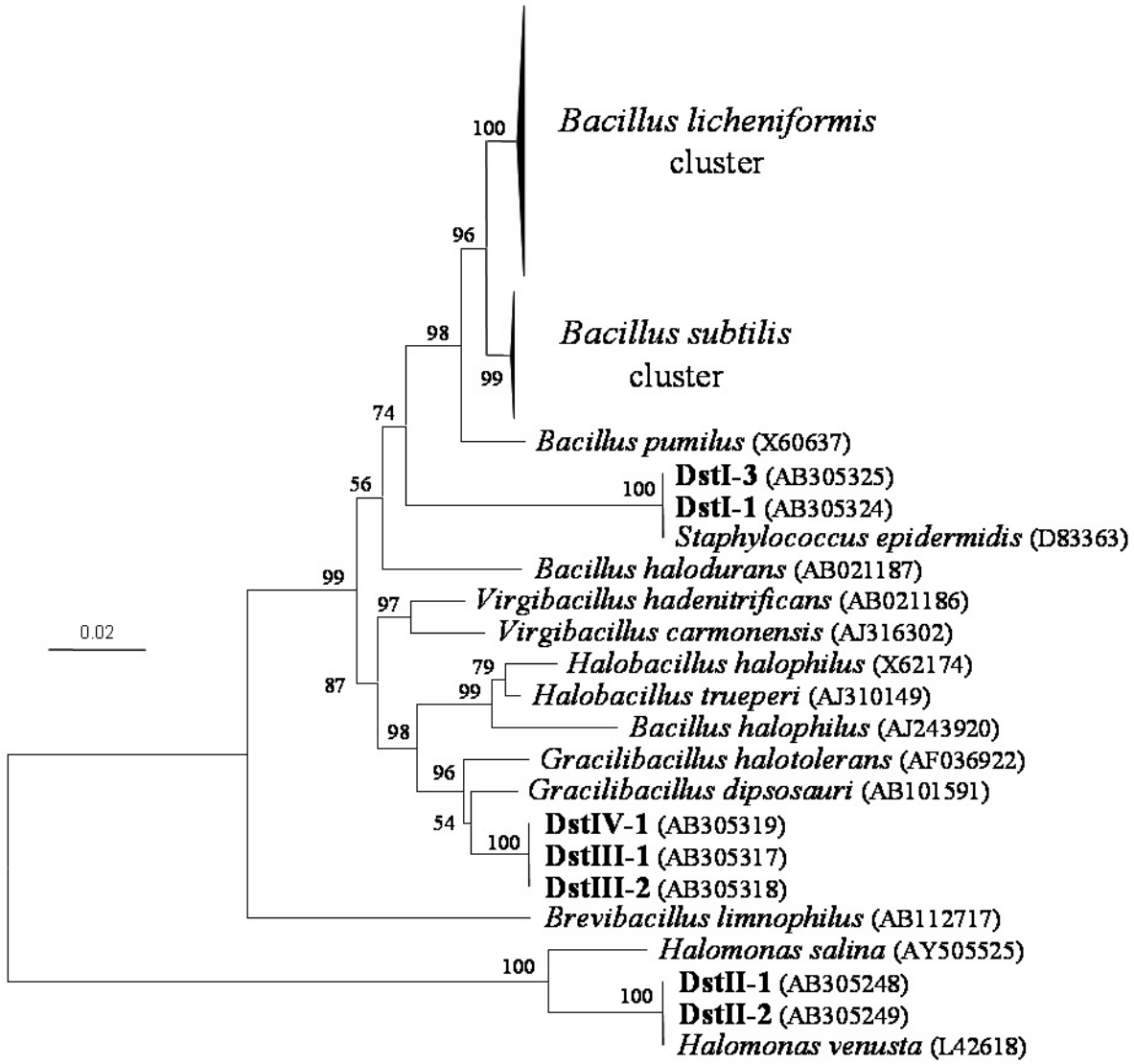
406 [b] references taken from sequence databases, GenBank/EMBL/DDBJ

Fig. 1



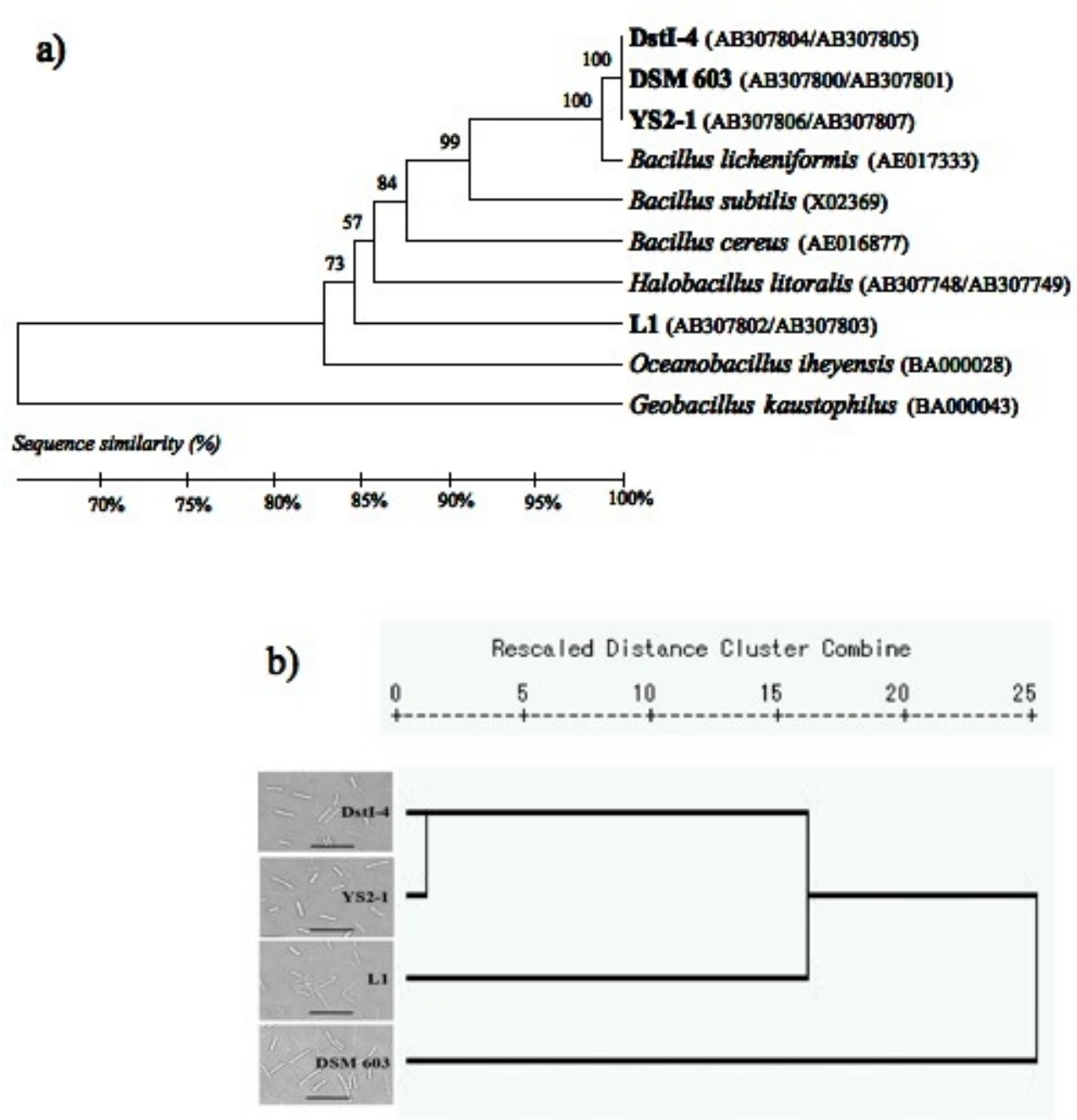
A meteorological map showing areas in Korea and Japan impacted by a yellow dust which originated in the Gobi Desert on March 31, 2007 (adapted from a predicted map of the Japan Meteorological Agency). *Filled circles* Sites affected by yellow dust. The sampling sites, Higashi-Hiroshima and Dunhuang, are indicated

Fig.2



Neighbor-joining tree showing phylogenetic clusters of dust-borne and desert-related isolates with other selected bacteria. Bootstrap values of 1000 replications greater than 50% are shown at the *nodes* of tree. Names in *bold* indicate strains identified in this study. 16S rRNA gene sequence accession numbers are in *parenthesis*. *Scale bar*: 0.02 nucleotide substitution per site. *Bacillus licheniformis* cluster includes isolates DstI-4 (AB305269), YS1-5 (AB305271), YS2-1 (AB305272), YS2-2 (AB305273), YS2-3 (AB305274), YS2-4 (AB305275), YS2-5 (AB305276), L1 (AB305265), and reference strains DSM 603 (DQ081997), DSM 13 (NC006322), CICC 10107 (DQ112220), K19 (DQ351932), BCRC 15413 (DQ993676), CICC 10181 (AY842871), CCBAU 10724 (EF405624); *Bacillus subtilis* cluster includes isolates DstI-2 (AB305268), YS1-4 (AB305270), AU30 (EF032678), clone B609 (DQ290002), Ni36 (DQ643186), GCNB5 (DQ834373), CICC 10034 (AY881640)

Fig.3



a A Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree showing a monophyletic line of isolates DstI-4. YS2-1 and strain DSM 603 were compared to other related bacteria based on composites of sequences of the *gyrB* and *parE* genes. Bootstrap values of 1000 replications greater than 50% are placed at the *nodes*. *Scale bar* indicates nucleotide sequence similarity (%). **b** An average linkage dendogram inferred from similarity coefficients (S_{SM} and S_J) of the numerical taxonomic analysis based on data of 150 physiological tests. Names and cell micrographs (*bar*: 10 μm) of strains are noted in the end of the dendogram