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UV-resistant bacteria isolated from upper troposphere and lower stratosphere

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Abstract Five bacterial strains have been isolated from dust samples collected from the upper troposphere and lower stratosphere during several aircraft flights. Most of them displayed much higher resistance to ultraviolet radiation (254 nm) than surface airborne isolates. The role of UV radiation combined with other conditions to determine survivability of bacterial species in the upper atmosphere is discussed. Two strains from the upper atmosphere (ST0316 and TR0125) exhibited extreme UV resistance and tend to form cell clumps or aggregates. Forming cell aggregation might be a strategy to enhance their survivability in the harsh conditions such as high dosage of UV at high altitude.

Introduction

Within the UV spectrum of solar irradiation, UVA (315-400 nm) predominates at the earth surface with small amounts of UVB (280-315 nm). UVC (<280 nm) radiation and high levels of UVB are harmful to living organisms. UVC and approximately 90% of UVB are absorbed by ozone, water vapor, oxygen and carbon dioxide in the atmosphere (World Health Organization, 2002), and do no harm the organisms on Earth's surface. At higher altitude, UV exposure is severe due to the thinner atmosphere. With every 1,000-meter increase in altitude, UV levels generally increase by 10 to 20%, depending on latitude, weather, time of day and year (Blumthaler *et al*., 1997; Seckmeyer *et al*., 1997; Schmucki and Philipona, 2002; World Health Organization, 2002). The amount of cosmic radiation also increases with the increase in altitude (Kendall, 2005), but cosmic rays are much less effective than UV radiation in causing biological damage (Galante and Horvath, 2007).

Microorganisms may be vertically transported into upper atmosphere via various mechanisms (Griffin, 2005). The relatively small size and low density of microorganisms like bacteria or spores permit them to remain airborne for long periods before they sediment to the ground (Atlas and Bartha, 1997). Microorganisms that survive in the high atmosphere are presumed to be resistant to ambient radiation. However, there was little evidence that UV radiation or cosmic ray plays a role to determine the survivability of microbial species in the upper atmosphere.

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The upper atmosphere remains to be a little explored area for microbiology, mainly due to the lack of sampling opportunity. To date, few samples have been collected in the higher atmosphere and the microorganisms in these samples studied. The earliest investigations were performed in the late 1800's and early 1900's. The organisms collected included fungi and spore-forming pigmented bacteria (Cristiani, 1893; Harz, 1904). Later studies reported the recovery of fungi, and spore-forming bacteria such as Bacilli (Rogers and Meier, 1936; Bruch, 1967; Wainwright *et al*., 2003; Griffin, 2005), non-spore-forming Micrococci and Mycobacterium (Fulton, 1966; Bruch, 1967; Imshenetsky *et al*., 1978; Griffin, 2008). In this study, we investigated bacterial isolates from the upper troposphere and lower stratosphere and atmosphere at the ground level, and show the higher UV resistance of the isolates from the upper atmosphere than those from surface air. Two Deinococcus-related isolates, particularly the stratospheric isolate, tended to aggregate in culture medium and exhibited extreme UV resistance as D. radiodurans did.

Materials and Methods

Air dust sampling

Dust samples in the atmosphere were collected using an air dust sampler equipped on an aircraft (Gulfstream-2, Diamond Air Service, Japan). Air outside the aircraft was introduced from the opening of a stainless steel tube (Fig. 1A). The tube was held by a support fixed on the outer wall of the aircraft, and opened to the forward (ram) direction of the aircraft at about 15 cm apart from the outer surface of the aircraft. The stainless steel tube (Fig. 1A) was connected to the inlet of the air dust sampler with a Teflon tubing in the cabin. Two types of dust samplers (Japan Machinery Co., Japan) were used. The original (ADS1) and the modified type (ADS2, Fig. 1) of air dust sampler had the same basic mechanism, except that ADS1 was equipped with only one filter holder and one pump. Air was introduced from outside

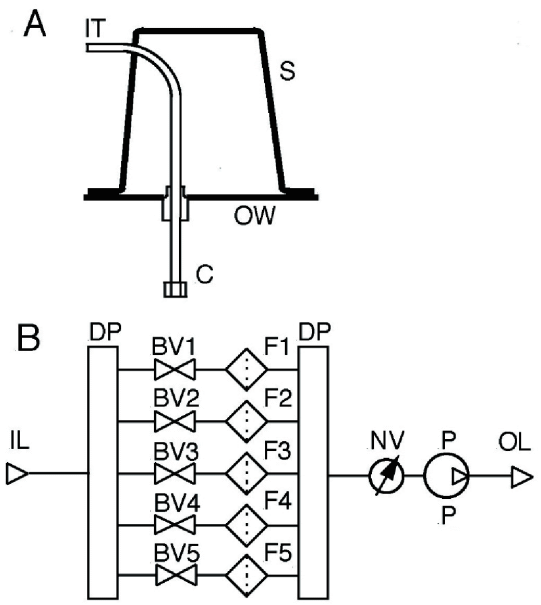

Fig. 1. Devices used for air borne dust sampling on aircraft. (A) Structure of the sampling tube attached to the outer wall of the aircraft. OW, outer wall of the aircraft; S, support; IT, intake of sampling tube; C, connector for Teflon tube. Flow diagram (B) of an air dust sampler (ADS2). IL, inlet connected to the Teflon tube; DP, dividing pipe; BV1-5, ball valves; F1-5, filter holders; NV, needle valve; P, pump; OL, outlet to Teflon tube connected to the exhaust apparatus of the aircraft. A needle valve was used to control flow rate. Airflow through filter was controlled individually by each ball valve.

of the aircraft by using a reciprocal oil-less vacuum pump (71R634, Gast Manufacturing Inc., USA). The flow rate was controlled by a needle valve, operated at a rate of 3 to 23 L min^{-1} monitored by a mass flow meter (3920E-AIR, Kofloc Ltd., Japan) and recorded on a chart recorder.

Dust samples were collected on membrane filters (type HA, diameter 47 mm, pore size 0.45 μm, Millipore USA) using ADS1 (Jan. 1999, Mar. 1999) and ADS2 (Nov. 2000) above the central part of the Main Island of Japan (Table 1). Prior to sampling, the filters were sterilized in an autoclave and placed in the sterilized filter holders on a clean bench. The filter holders were set on a dust sampler installed in the aircraft. The vacuum pump was operated with a test filter to clean the inside of the tubing before each flight or after the preceding flight in 1999. The ball valve was opened and the pump was operated at each altitude indicated in Table 1. Instead of the test filter, the first filter was used to check and clean possible contamination inside of the tubing during sampling in 2000 from 0 to 1.2 or 3 km above ground. Subsequently the other four filters were sequentially used to collect samples. Control dust samples at the ground level were collected on the same type membrane filters using a commercial air dust sampler ABB-1 (Shibata Scientific Technology, Japan) or ADS1. After sampling, the filters were detached from the holders and wrapped with sterile aluminum foil on a clean bench, and stored at 4 °C until analysis.

The transition altitude from troposphere to stratosphere was determined by the observed reversal of temperature gradient during ascending flight. Atmospheric temperature decreases in troposphere, and increases above the tropopause during ascent (Schneider, 2005).

Bacterial isolation and identification

Filters were placed on plates of Bacto mTGE medium (Difco, USA) and incubated in the dark at 30 °C. The colonies developed on the filters were cultured in mTGE medium. Unless stated otherwise, cultures grown in mTGE medium at 30 °C were used in the experiments described below. Glycerol (0.5 %) and KH_2PO_4 (0.1 mM) were used to supplement mTGE broth for growth of isolate ST0316. Nutrient agar no. 2 (peptone 10.0 g, meat extract 10.0 g and NaCl 5.0 g per liter, pH 7.0-7.2) recommended by the Japan Collection of Microorganisms was also used as culture medium for two reference strains (*D. radiodurans* JCM 6273 and ATCC 13939). Fifteen grams of agar per liter were added to obtain solid media.

An Olympus BX-FLA microscope (Olympus, Japan) was used to assess morphology, motility and spore formation. For scanning electron microscopy (SEM), cells were deposited on a plastic sheet coated with poly-(L-lysine) and fixed with 2% glutaraldehyde. The preparations were then dehydrated in ethanol, critical point dried, coated with metallic gold and examined in a low vacuum JSM-5800LV microscope (JEOL, Japan) operated at 15 kV.

Genomic DNA was extracted and purified with a FastDNA kit (BIO101, Vista, USA). A 16S rDNA fragment (corresponding to *E. coli* nucleotides 10-1500) was amplified by PCR with primers EU 10F (5'-AGAGTTTGATCCTGGCTCAG-3') and EU 1500R (5'-GGTTACCTTGTTACGACTT-3') (Takami *et al*., 1999). The PCR products were either cloned using a TOPO TA Cloning Kit pCR2.1-TOPO (Invitrogen, Carlsbad, USA) or were sequenced directly. The sequences were determined using an ABI Big Dye Terminator Cycle Sequencing FS Ready Reaction kit on an ABI PRISM 377 DNA Sequencing System (Perkin Elmer, USA). The 16S rDNA sequence of closely related species were

TABLE 1. Upper atmospheric samples and their viable microbial load

Date or location	Altitude (km)	Atmospheric pressure (kPa)	Atmospheric temperature (°C)	Volume of air a (L)	Viable counts [a](CFU)
1999 (ADS1) [b]	Stratosphere				
May 12	11 ~ 12	23 ~ 19	-56	63	0
May 16	10 ~ 12	26 ~ 19	-50 ~ -56	48	1
May 17	11 ~ 12	23 ~ 19	-56	78	0
Total				189	1 (0) [c]
1999 (ADS1)	Troposphere				
Jan. 25	0.8 ~ 5.8	92 ~ 48	1 ~ -28	328	1
Jan. 26	4.6 ~ 10	57 ~ 26	-20 ~ -50	287	1
Total				615	2 (0)
2000 (ADS2)	Troposphere				
Nov. 3-1	0 ~ 1.2	101 ~ 88	15 ~ 7	10	0 (1)
Nov. 3-2	1.2 ~ 7.8	88 ~ 37	7 ~ -36	184	1
Nov. 3-3	7.8 ~ 12.2	37 ~ 19	-36 ~ -56	45	1
Nov. 3-4	12.2	19	-56	63	0
Nov. 3-5	12.2 ~ 1.2	19 ~ 88	-56 ~ 7	365	0 (1)
Nov. 3 total				657 [d]	2 (1) [d]
Nov. 7-1	0 ~ 3.0	101 ~ 70	15 ~ -5	35	1
Nov. 7-2	3.0 ~ 7.8	70 ~ 37	-5 ~ -36	251	0
Nov. 7-3	7.9 ~ 12.2	37 ~ 19	-36 ~ -56	63	0
Nov. 7-4	12.2 ~ 12.3	19	-56	60	0 (1)
Nov. 7-5	11.6 ~ 0.9	21 ~ 91	-56 ~ 9	331	0
Nov. 7 total				705 [d]	0 (1) [d]
Nov. 8-1	0 ~ 3.0	101 ~ 70	15 ~ -5	44	0 (1)
Nov. 8-2	3.0 ~ 6.9	70 ~ 42	-5 ~ -30	217	0
Nov. 8-3	6.4 ~ 12.2	45 ~ 19	-27 ~ -56	69	0
Nov. 8-4	12.2	19	-56	56	0
Nov. 8-5	12.2 ~ 2.8	19 ~ 72	-56 ~ -3	180	0 (1)
Nov. 8 total				522 [d]	0 (1) [d]
2000 total				1884 [d]	2 (3) [d]
Airport (ABB-1)				4060	4 (33)
Univ. campus					
(ADS1)				1870	1 (5)
(ABB-1)				6840	18 (42)

[a] Corresponding mass volume of one liter at 1 atm.
[b] Type of sampler used.
[c] Figures in parentheses indicate numbers of fungal colonies.
[d] The data for volume and CFU for the first filters (Nov. 3-1, Nov. 7-1 and Nov. 8-1) were not included in the total values.

searched using the BLAST algorithm (blastn) at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). The 16S rDNA sequences were deposited into the NCBI database under the following accession numbers: AB087287 (strain ST0316), AB087288 (strain TR0125), AB087854 (strain TR0126), AB088145 (strain TR1103-2), and AB089198 (strain TR1103-3).

UV survival test

UV survival experiments were carried out as described by Miller (1992) and Arrage *et al*. (1993a). Bacterial cells in exponential phase were harvested, washed, and suspended in 20 mM KH_2PO_4 buffer (pH 7.0). Cells of isolate ST0316, which formed aggregates during growth, were dispersed with a plastic disposable homogenizer prior to exposure to UV (254 nm) at a rate of 290 μW cm^{-2} from a GL-15 germicidal lamp (National, Japan) for different lengths of time and plated on triplicate TGE plates after serial dilution. D. radiodurans JCM 6273 and ATCC13939 were plated on nutrient no. 2 agar plates. Isolate ST0316 was plated on mTGE plates supplemented with glycerol (0.5%) and KH_2PO_4 (0.1 mM). Other isolates were plated on mTGE plates without the supplementation. Plates were wrapped in aluminum foil during incubation. Percent survival was calculated as the colony counts of irradiated cells divided by those of the non-irradiated control cells multiplied by 100. D-values (D_{37} and D_{10}) were calculated from survival curves (Arrage *et al*., 1993a).

Results

Descriptions of samples and colony counts obtained from the samples are listed in Table 1. One bacterial colony (ST0316) was obtained from a sample collected from the stratosphere in 1999. No colonies grew from other stratospheric samples. Four bacterial colonies (TR0125, TR0126, TR1103-2, TR1103-3) were recovered from the tropospheric samples. The bacterial and fungal colonies detected on the first filter used in sampling in the year 2000 were not included in the analysis below. Colony forming units (CFU) per cubic meter of air (corresponding mass volume at 1 atmospheric pressure) in the stratosphere were 5.3. CFU per cubic meter of air in the troposphere were 3.3 in the year 1999 and 1.1 in the year 2000. Bacterial density in the surface air was between 0.53 and 2.6 CFU m^{-3} at the Nagoya airport (Nagoya, Japan) and on the Tokyo University of Pharmacy and Life Science campus (Tokyo, Japan) respectively. Whereas many fungal colonies were observed in the surface control samples, only three fungal colonies were obtained from samples collected in the high atmosphere.

The 16S rDNA sequences of orange-red isolates ST0316 and TR0125 showed the greatest similarity, 93% and 96%, respectively, to that of *D. apachensis*.

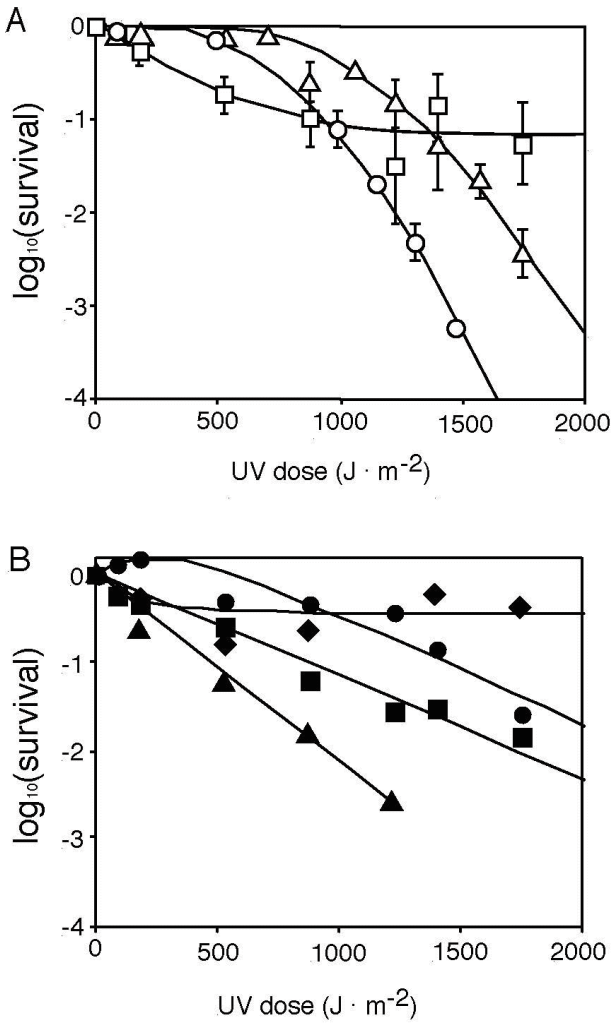

Fig. 2.
A. UV survival curves for bacterial isolates ST0316 (open square), TR0125 (open triangle) and for D. radiodurans ATCC 13939 (open circle). Standard bars represent standard errors of the mean. The data for ST0316 (open square) represent the average of the four measurements shown in panel B.
B. UV survival curves from four independent experiments (closed symbols) for the bacterial isolate ST0316.

The Blast search indicated that the 16S rDNA sequence similarity values were highest between the two isolates and reported *Deinococcus* species. The 16S rDNA sequences of other three upper-atmospheric isolates share up to 98% similarity to other described species: TR0126 to *Streptomyces* species (near-complete 16S rDNA sequences were compared); TR1103-2 to *Bacillus* species (about 0.7 kbp of the 5' end of the 16S rDNA sequences were compared); TR1103-3 to *Paenibacillus* species (about 0.9 kbp of the 3' end of the 16S rDNA sequences were compared).

TABLE 2. UV resistance of bacteria isolated from air dust samples and of *Deinococcus radiodurans* strains.

Strain	Altitude (km) or site	D_{37} [a] ($J\ m^{-2}$)	D_{10} [a] ($J\ m^{-2}$)	No. of exp.
ST 0316	10-12	300 (170 - 900)[b]	1000 (500 – 1500) [b]	4
TR 0125	0.8-5.8	1000 ± 150 [c]	1200 ± 130 [c]	4
TR 0126	4.6-10	310 ± 190	710 ± 400	4
TR 1103-2	1.2-7.8	48 ± 7	97 ± 12	3
TR 1103-3	7.8-12.2	74 ± 14	140 ± 48	3
AP 0126-1*	Airport	39	98	1
AP 0126-2*	Airport	23	60	1
AP 0126-3*	Airport	23	61	1
TU 0614-1*	University campus	19	45	1
TU 0614-2*	University campus	53 (± 14)	91 (± 26)	2
D. radiodurans JCM 6273 [d]		570 ± 170	860 ± 200	3
D. radiodurans ATCC 13939 [d]		700 ± 69	870 ± 56	3
D. radiodurans ATCC 13939 [e]		400 ±130	910 ± 290	
D. radiodurans ATCC 13939 [f]		338.0± 5.3	553.1± 8.7	

[a] D-values are defined as the UV dose which reduced a cell population to a specified percentage of the original number of cells. The D-values were calculated from the regression line of the exponential slope of the survival curves as described in Arrage *et al*. (1993a).

[b] Range of values.

[c] Mean±SEM (or±max-mean).

[d] Nutrient agar no. 2 plates were used for estimation.

[e] Arrage *et al*.

[f] Gascon *et al*..

* The strains found most resistant to UV irradiation among 23 ground-level bacterial isolates (Table 1).

The UV resistance of *D. radiodurans* ATCC 13939 was not dependent on the kind of media used (TGE agar plates with or without glycerol and KH_2PO_4, or nutrient agar no. 2 plates) (data not shown). D_{37} values estimated for *D. radiodurans* JCM 6273 and ATCC 13939 were 570 and 700 J m^{-2}, respectively, similar to previously reported values (Arrage *et al*., 1993b; Gascon *et al*., 1995) (Table 2). Two upper-atmospheric bacterial isolates (ST0316 and TR0125) exhibited extremely high UV-resistance (Fig. 2A). D-values for isolate TR0125 were higher than for *D. radiodurans*, while the D-values of the isolate ST0316 were comparable to those of *D. radiodurans* (Table 2). Survival of isolate ST0316 was found to vary (Fig. 2B), possibly due to the tendency of cells to form aggregates which were observed in spite of the use of a homogenizer. The CFU per plate varied up to 10-fold using triplicate plating for this isolate. ST0316 exhibited a higher survival rate than *D. radiodurans* in three of four independent trials. TR0126 exhibited relatively high UV-resistance. Other upper-atmospheric isolates exhibited moderate UV resistance. D-values for the most UV-sensitive isolate from the upper atmosphere (TR1103-2) were comparable to those of the most UV-resistant strain isolated from the Earth surface layer (AP0126-2).

Discussion

The upper-atmospheric isolates

Imshenetsky *et al*. (1977, 1979) reported the importance of pigment for higher UV resistance of most isolates in their upper-atmospheric samples and concluded the predominance of pigmented microorganisms as the consequence of natural selection caused by UV radiation. However, their UV-sensitive and pigmentless isolate *Micrococcus albus* was not consistent with their conclusion (Imshenetsky *et al*., 1979). In the current study, all upper-atmospheric bacterial isolates from the samples collected during several flights, exhibited higher resistance to UV radiation than surface-level bacterial isolates (Table 2). Although the mTGE broth we used for the isolation may not have supported growth of all the bacterial species present in the air samples tested, it is not a selective medium for UV-resistant bacteria. Upper-atmospheric isolates TR1103-

2 and TR1103-3 exhibited comparable UV-resistance to the most UV-resistant isolate from surface atmosphere. *Deinococcus*-related isolates (ST0316 and TR0125) exhibited extreme UV resistance common to described *Deinococcus* strains (Weon *et al*., 2007). The high UV-resistant isolate TR0126 is a *Streptomyces*-related strain. Although *Streptomyces* species are generally not considered to be UV-resistant, a relatively high UV-resistant strain of *S. coelicolor* A3(2) has been reported (Puglia and Cappelletti, 1984). The isolate TR0126 is possibly a UV-resistant strain of *S. coelicolor* or a *Streptomyces* mutant of high UV resistance. These three isolates from the upper atmosphere exhibited greater UV resistance than any other bacterial isolate in this study. To our knowledge, the upper atmosphere is the sole natural environment from which such high proportion of UV-resistant bacteria was isolated.

The role of UV on selecting the survivors

The lower atmosphere is constantly replenished by various microorganisms from soil, water and other sources (Lighthart and Shaffer, 1995; Shaffer and Lighthart, 1997; Griffin *et al*., 2003). Once becoming airborne, microorganisms face the problem of survival. Desiccation is a major cause for microorganisms to lose viability, particularly in the lower atmosphere during the day (Atlas and Bartha, 1997). However, the dried microorganisms at low temperature or vacuum (reduced pressure) conditions survive better than at room temperature or normal atmospheric pressure (Miyamoto-Shinohara *et al*., 2006). The freezing-drying and vacuum-drying conditions are often used for the storage of microorganisms (Potts, 1994). Accordingly, the lower temperature and pressure in the upper atmosphere are favorable for microbial survival. If UV radiation were not present in the atmosphere, the upper atmospheric condition would exert less pressure for microbial survival than the surface atmospheric condition, and permit the survival of various microorganisms that can survive in the surface atmosphere. In other words, UV-sensitive bacteria could also have been isolated from our upper-atmospheric samples, if they were not exposed to UV radiation.

In the surface atmosphere we have isolated many clones of low UV-resistance (Table 2). Apparently, UV resistance is not important for microbial survival in the surface atmosphere. However, the lethal effect of UV radiation is severe in the upper atmosphere. UV levels are elevated in the upper atmosphere as discussed in Introduction. Although the low temperature and pressure in the upper atmosphere is advantageous for microbes to survive, the harmful effect of UV can be enhanced by the conditions. Bacteria such as *E.coli, Bacillus* and *Deinococcus* species have been reported to be more sensitive to UV radiation under vacuum or/and under desiccating conditions (Saffary *et al*., 2002; Diaz and Schulze-Makuch, 2006). UV-induced killing, therefore, is very likely the major cause for microorganisms to lose viability in the upper atmosphere. It is consistent with the high proportion of UV-resistant bacterial isolates in the current study.

Deinococcus *isolates and cell aggregation*

The isolates of the genus *Deinococcus* have been described from diverse surface habitats (Weon *et al*., 2007) to the deep-sea biosphere (Kimura *et al*., 2003). The airborne *Deinococcus* was firstly found from hospital air contaminant (strain SARK) (Murray and Robinow, 1958), and isolated later from indoor air dust (Christensen and Kristensen, 1981) and recently from island surface air (Weon *et al*., 2007). The two Deinococcus strains isolated in the current study are the first documentation of the genus *Deinococcus* from upper boundary of the biosphere, the upper atmosphere.

It is noteworthy that the UV resistance of the *Deinococcus* isolates ST0316 and TR0125 was similar to that of *D. radiodurans*. The significantly higher survival rate after a large dose of UV light (Fig. 2) can be attributed to shielding of UV light by cells on the outside of the observed cell aggregates. The colonies of these strains are orange-red pigmented. These pigments may exert the protection effect against UV light. Cell aggregation is viewed as a survival strategy against UV irradiation (Wainwright *et al*., 2003) and could be an explanation for the occurrence of UV-sensitive *M. albus* in previous upper-atmospheric sample. The slow growth of the isolates ST0316 in culture medium might also relate to strong cell aggregation. Although we tried disposable plastic homogenizer to disperse cell aggregates, which didn't affect cell viability, this treatment was only partially successful. We assume that the tendency of ST0316 cells to form strong aggregate was the cause of the variable CFU/ml observed for this isolate. Such tendency was not observed for reference strains *D. radiodurans* JCM 6273 and ATCC 13939. While isolate TR0125 did not form cell aggregates in culture medium, its cell pellet was formed during the same centrifugation step, which was much more difficult to be resuspended than that of *D. radiodurans* ATCC 13939. Extensive cell clumps in liquid culture have been reported for previously described *Deinococcus* species, the desiccation tolerance of which could not be accurately determined (Rainey *et al*., 2005). The mechanism of cell aggregation in these *Deinococci* is not well known. Type IV pili contributed to the attachment of sessile cells of *D. geothermalis* to the abiotic surface and to the neighboring cells, but they were absent from the planktonic cells (Saarimaa *et al*., 2006). Such thread-like pili were not observed for the cells of isolates ST0316 and TR0125 (Fig. 3). The strains will be characterized elsewhere in details.

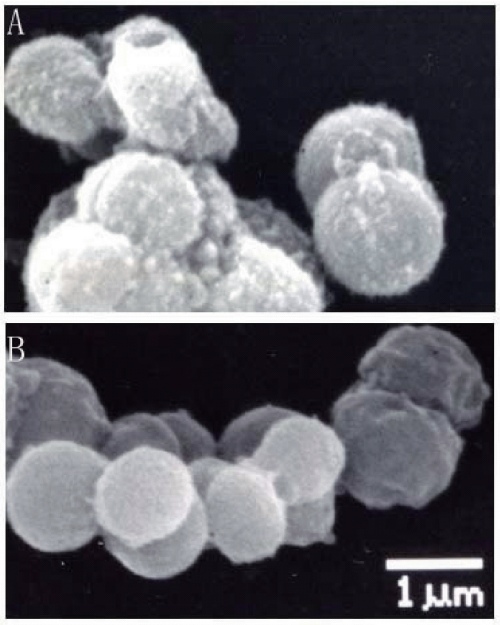

Fig. 3. SEM photos of the cells of upper-atmospheric isolates ST0316 (A) and TR0125 (B).

Conclusion

The important role of UV radiation on selecting bacterial species in the upper atmosphere was indicated by UV-resistant bacterial isolates from the samples collected during several aircraft flights. Cells of the first documented *Deinococcus* strains from the upper atmosphere tend to form clumps or aggregates, and exhibited extreme UV resistance. Forming cell aggregates might be a strategy to enhance their survivability in the harsh atmospheric conditions.

Acknowledgments

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