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Seasonal Change in Bacterial Flora and Biomass in Mountain Snow from the Tateyama Mountains, Japan, Analyzed by 16S rRNA Gene Sequencing and Real-Time PCR

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The bacterial flora and biomass in mountain snow from the Tateyama Mountains, Toyama Prefecture, Japan, one of the heaviest snowfall regions in the world, were analyzed by amplified ribosomal DNA restriction analysis followed by 16S rRNA gene sequencing and DNA quantification by real-time PCR. Samples of surface snow collected in various months during the melting season contained a psychrophilic bacterium, *Cryobacterium psychrophilum*, and two psychrotrophic bacteria, *Variovorax paradoxus* and *Janthinobacterium lividum*. Bacterial colonies that developed in an in situ meltwater medium at 4°C were revealed to be *V. paradoxus*. The biomasses of *C. psychrophilum*, *J. lividum*, and *V. paradoxus*, as estimated by real-time PCR, showed large increases during the melting season from March to October (2.0×10^5-fold, 1.5×10^5-fold, and 1.0×10^4-fold increases, respectively), suggesting their rapid growth in the surface snow. The biomasses of *C. psychrophilum* and *J. lividum* increased significantly from March to April, reached a maximum in August, and dropped at the end of the melting season. In contrast, the biomass of *V. paradoxus* did not increase as rapidly during the early melting season but continued to increase from June until October. The differences in development observed among these bacterial species suggest that their growth was promoted by different nutrients and/or environmental conditions in the snow. Since these three types of bacteria have also been reported to be present in a glacier in Antarctica and a Greenland ice core, they seem to be specialized members of the snow biota that are distributed in snow and ice environments in various parts of the world.

Snow and ice environments contain various microorganisms, such as snow algae and bacteria. Recent studies have revealed relatively closed ecosystems in glaciers and other environments of permanent snow and ice that harbor a variety of cold-tolerant organisms (13, 14, 34). In glacier ecosystems, microorganisms play important roles. For example, snow algae serve as primary producers that sustain heterotrophic communities on glaciers, including insects, copepods, ice worms, fungi, and bacteria (1, 8, 10, 12, 13). Many reports have been published on the taxonomy (10, 17–20), physiology (9, 22), and ecology (10, 29, 30, 38) of snow algae. However, information on snow and ice bacteria is still very limited. Although many researchers have reported the presence of considerable numbers of bacteria in snow and ice in glaciers (13–15, 32) and in perennial snow patches (16, 35), which suggests their growth in snow and ice, these bacteria were not taxonomically identified because it was impossible to identify bacterial species exclusively from their morphology. Moreover, bacterial growth in snow and ice has not been verified. In some recent studies of microorganisms contained in ice cores from the polar regions (2, 24, 37), species identification of bacteria was conducted by PCR amplification and 16S rRNA gene analyses. However, all of these studies postulated that these bacteria were originally airborne or lived in the lake below the ice sheet. In addition, because many studies (3–5, 21, 25, 26, 40, 41) have examined bacteria isolated from ice samples on nonselective media, such as nutrient broth, the bacterial species identified in these studies seem unlikely to have been members of the snow and ice biota adapted to cold and extremely oligotrophic conditions. Therefore, we still do not know which groups of bacteria grow in snow and ice environments.

An understanding of the ecology of bacteria found in snow and ice is also important for studies of the effects of the microbial biota on glacial melting and for studies of past environments by ice-core analyses. Blooms of algae and bacteria can reduce the surface albedo of snow and ice and significantly affect their melting (15, 32, 35). For example, the surface albedo of some Himalayan glaciers is significantly reduced by a large amount of dark-colored biogenic material (cryoconite) derived from snow algae and bacteria (15, 32). This material increases the melting rates of the surfaces as much as threefold (15). Thus, it was suggested that microbial activity in snow and ice affects the mass balance and fluctuation in size of the glacier. Ice cores, which are well-preserved proxies of past environmental conditions, are usually analyzed only by physical and chemical methods. However, the presence of microorganisms such as snow algae in ice cores can also be used to interpret past environmental conditions (38, 39). Thus, ecolog-

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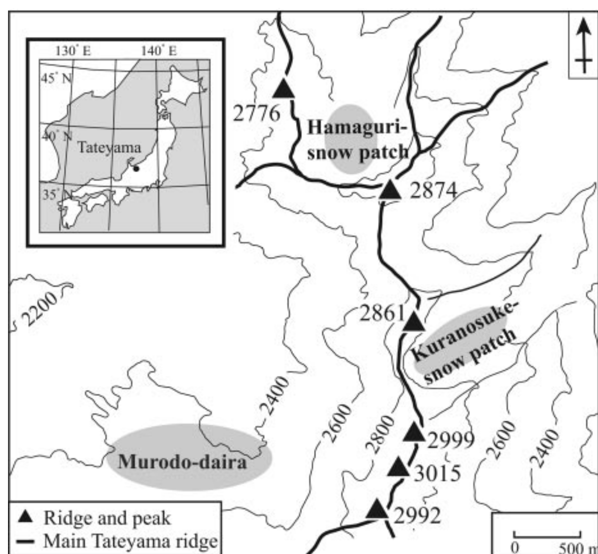

FIG. 1. Location of the Tateyama Mountains, Toyama Prefecture, Japan, and map of the study area showing the sampling sites. The contour interval is 200 m.

ical studies of bacteria and algae in snow and ice are important not only biologically but also glaciologically.

For this study, in order to identify bacterial species growing in the snow, we analyzed bacterial DNAs extracted from mountain snow samples collected in the Tateyama Mountains, Toyama Prefecture, Japan, by amplified ribosomal DNA restriction analysis (ARDRA) followed by 16S rRNA gene sequencing.

We also isolated bacteria by culturing snow samples with an in situ snowmelt medium at 4°C and identified them by sequencing their 16S rRNA gene amplicons. We then examined their growth in snow by analyzing seasonal changes in the biomass of each bacterial species in the surface snow, as estimated by DNA quantification with real-time PCR. Since the bacterial biomass in surface snow is affected not only by bacterial growth but also by other factors, such as the supply of bacteria from the atmosphere, condensation by snow melting, and the effect of meltwater washout, we also examined the effects of these factors.

Our results indicated that the surface snow of this area contains one psychrophilic bacterium and two psychrotrophic bacteria. Our data also strongly suggested that these bacteria grow in the surface snow during the melting season. Records of their distribution suggested that these bacterial species are specialized members of the snow biota that are distributed in snow and ice environments in various parts of the world.

MATERIALS AND METHODS

Snow samples. Snow samples were collected at Murodo-daira (36°34′N, 137°36′E, 2,450 m above sea level [a.s.l.]) and from nearby perennial snow patches (the Kuranosuke snow patch, located at 36°35′N, 137°37′E, 2,600 m a.s.l., and the Hamaguri snow patch, located at 36°36′N, 137°37′E, 2,700 m a.s.l.) in the Tateyama Mountains, Toyama Prefecture, Japan (Fig. 1). The Tateyama Mountains, located near the west coast of Honshu Island and facing the Sea of Japan, are well known as one of the heaviest snowfall regions in the world. In Murodo-daira, snow accumulation during winter often exceeds 6 m (23), and many snow patches remain until midsummer.

In order to identify bacteria in the surface snow of this area, we collected samples of the surface snow at Murodo-daira on 10 March 2000 and at Hamaguri sekkei on 10 June and 1 August 2000. Surface snow (1.5 cm deep) was collected with a sterilized stainless steel scoop from randomly selected points (30 by 30 cm) at the sampling site (5 by 5 m) throughout the study. Snow samples were stored in 50-ml sterile plastic conical tubes (Iwaki, Tokyo, Japan) or sterile plastic bags (Whirl-pak bags; Nasco, Fort Atkinson, Wis.) at −20°C until required. The same precautions against contamination and the same types of containers were used for all snow sampling for this study. In order to investigate the level of the original bacterial biomass in the snow before melting, we collected snow samples from the wall of a snow pit (6 m deep) at Murodo-daira on 20 March 1998. We collected snow samples (50 cm^3 each) from every snow layer with at least a 10-cm thickness from the surface to the bottom. The temperature of each snow layer ranged from −9 to −7°C.

In order to investigate seasonal changes in the total and specific bacterial biomasses in the surface snow, we sampled surface snow almost every month from March through October 1998 at Murodo-daira (March) and Kuranosuke sekkei (April, May, June, July, August, and October). We collected the March sample at Murodo-daira because we could not access Kuranosuke sekkei at that time.

In March, the condition and contents of the surface snow at Murodo-daira were considered to be almost the same as those of the snow at Kuranosuke sekkei and Hamaguri sekkei because the snow temperature was well below the freezing point (−7 to −9°C) and no snowmelt had yet occurred at Murodo-daira. The snow at the sampling site of Murodo-daira in March appeared to be homogenous due to continuous snowfall. Surface snow (a total of approximately 2 liters) was collected from a randomly selected point. In contrast, the appearance of the surface snow at the sampling site of Kuranosuke sekkei during the other months was not homogenous, but rather was a mosaic of brown parts and white parts. Therefore, the collection of surface snow was performed at eight randomly selected sites (90 cm^2, 1.5 cm deep) from April to August and at three sites in October. To calculate the bacterial concentration and the algal biomass per unit area, we measured the exact surface area of the collection site on the snow.

Dust trap samples. The air over the sampling sites was tested with a dust trap to determine the quantity of bacteria and dust particles that were deposited in the snow from the atmosphere. We installed a simple dust trap on a rock beside the snow patch from May to October 1998. The dust trap was made with a wide-mouth (9-cm diameter) 500-ml plastic bottle containing 100 ml of glycerol with formalin (10% [wt/wt]). To avoid rain falling directly into the dust trap and to retain good ventilation, we equipped the bottle with a small cover, which was fixed with a wire 1.5 cm above the bottle mouth. We collected the liquid samples in the dust traps about every 2 weeks.

Microscopy. Bacteria and mineral particles (0.5 to 3.0 μm in diameter) in snowmelt and dust trap samples were concentrated by deposition on a membrane filter (0.22-μm pore size) (JGWP01300; Millipore, Tokyo, Japan). Bacteria deposited on the filter membrane were stained with DAPI (4′,6′-diamidino-2-phenylindole) before counting. An optical microscope (Nikon Optiphoto 2) was used to count the bacteria and mineral particles at a magnification of ×1,000. The quantity of the bacterial biomass was expressed as the cell concentration per unit area (cells per square centimeter).

Green algae and cyanobacteria in snowmelt samples were also collected by the filtering method described above. Algae and cyanobacteria were identified and counted according to their morphology under an optical microscope (Nikon Optiphoto 2) at a magnification of ×400. The quantities of the biomasses of green algae and cyanobacteria were expressed as the cell number per unit area (cells per square centimeter) or the total cell volume per unit area (cubic micrometers per square centimeter). The total cell volume was estimated from the mean cell volume and the cell concentration.

DNA extraction. A portion (1 liter) of the Tateyama snow sample from March 2000 was melted in a sterile plastic bag (Whirl-pak bag) in a clean booth, and the resultant water was filtered through a sterile 0.22-μm-pore-size filter unit (Millipore).

The membrane filter was transferred to a 15-ml sterile plastic tube (Iwaki) and subjected to DNA extraction by the method of Godon et al. (7) after being thoroughly washed with solution A (1 ml of 4 M guanidine thiocyanate in a 0.1 M Tris-HCl buffer [pH 7.5] and 150 μl of 10% *N*-lauroyl sarcosine).

Tateyama snow samples from June and August 2000 were melted as described above, and a portion of each (June sample, 100 ml; August sample, 50 ml) was transferred to a separate centrifuge tube from a well-stirred melt sample. Each tube was then centrifuged at 22,000 × *g* for 15 min to obtain pellets. The pellets were resuspended in a small amount of solution A (1 ml) and transferred to 1.5-ml safe-lock microcentrifuge tubes (Eppendorf, Tokyo, Japan) by use of a pipette. The samples were then subjected to DNA extraction as described above.

A portion (200 ml) of the Tateyama snow sample from March 1998 was melted and filtered as described above. Tateyama snow samples from April to October were melted as described above, and a portion (April and July samples, 20 ml; May sample, 40 ml; June sample, 30 ml; August sample, 10 ml; October sample, 1 ml) of each was centrifuged at 22,000 $\times$ *g* for 15 min to obtain pellets. The membrane filter and pellets for each sample were transferred to separate 1.5-ml safe-lock microcentrifuge tubes and subjected to DNA extraction with a FastDNA spin kit for soil (Qbiogene, Inc., Carlsbad, Calif.) in conjunction with a FastPrep FP120 instrument (Qbiogene, Inc.).

Amplification and cloning of bacterial 16S rRNA genes. A portion (2 μl) of each bacterial DNA sample was subjected to PCR amplification of the 16S rRNA gene by the use of *ExTaq* DNA polymerase (Takara, Kyoto, Japan) according to the manufacturer's directions. The PCR primers used for the amplification of the V6 to V8 regions of the 16S rRNA gene were 907F (5′-AAACTCAAATGAA TTGACGGG-3′) and 1407R (5′-ACGGGCGGTGTGTACAAG-3′). PCRs were performed under the following conditions: 3 min of initial denaturation at 95°C followed by 30 thermal cycles (95°C for 30 s, 56°C for 30 s, and 72°C for 45 s) and a final extension at 72°C for 10 min. The amplified products were isolated in gels and purified by use of a Qiaquick gel extraction kit (Qiagen, Hilden, Germany). They were then ligated into the pGEM-T vector (Promega, Madison, Wis.) according to the manufacturer's directions. *Escherichia coli* JM109 (Takara) was transformed with the cloning vector, and transformants were selected by blue-white selection on Luria-Bertani agar plates containing ampicillin (100 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside; 1 mg/plate), and IPTG (isopropyl-beta-D-thiogalactopyranoside; 2.38 mg/plate). Insertion of the appropriate size of DNA was determined by colony PCR amplification with a universal primer set (M13F and M13R) targeting both sides of the cloning vector. Colony PCRs were performed under the following conditions: 3 min of initial denaturation at 96°C followed by 30 thermal cycles (96°C for 30 s, 55°C for 20 s, and 72°C for 60 s) and a final extension at 72°C for 10 min.

ARDRA screening of clones and nucleotide sequence analyses. The clones from the Tateyama samples from March, June, and August 2000 were next subjected to ARDRA. The insert region, amplified as described above, was completely digested with three restriction enzymes, Sau3AI, HaeIII, and HhaI (Toyobo, Tokyo, Japan). The restriction fragments were analyzed by electrophoresis in a 2.2% agarose gel (wt/vol) in 1× Tris-borate-EDTA buffer. The gels were stained with ethidium bromide. Band patterns were compared in order to define bacterial operational taxonomy units.

The sizes of the clone libraries (numbers of *E. coli* cells) for samples from March 2000, June 2000, and August 2000 were 137, 120, and 165, respectively. ARDRA differentiated 24, 22, and 24 groups, respectively, in the libraries of the three samples. Sequence analysis was performed on several (one to three) randomly selected clones from each library.

Nucleotide sequencing of the inserted DNA fragment of each operational taxonomy unit was performed by use of a Big Dye Terminator cycle sequencing ready reaction kit and an automatic sequence analyzer (ABI model 310 or 3100; Applied Biosystems, Tokyo, Japan). The universal primers M13F and M13R were used as sequencing primers.

Homology searches were performed against the DDBJ/EMBL/GenBank nucleotide sequence database by use of the gapped FASTA program at the DDBJ website (http://www.ddbj.nig.ac.jp/Welcome-e.html).

The sequences obtained from nucleotide sequencing were compared with those in the databases, and those displaying similarities of $>$98% with known species were identified as corresponding species. Those displaying similarities of $<$98% with the sequences in the databases were considered unknown taxa.

Real-time PCR for quantification of specific bacteria. Real-time PCR was used to estimate the densities of specific bacteria in the snow samples from 1998. To obtain the densities of specific bacteria in snow samples, we established standard amplification curves with known numbers of specific bacteria and their threshold cycles. *Cryobacterium psychrophilum* JCM 1463 was obtained from the Japanese Collection of Microorganisms, RIKEN. *Janthinobacterium lividum* IAM 13948 and *Variovorax paradoxus* IAM 12373 were obtained from the Institute of Applied Microbiology Culture Collection, Institute of Molecular and Cellular Bioscience, University of Tokyo.

C. psychrophilum was cultured in Trypticase soy broth (Nissui, Tokyo, Japan) for 24 h at 4°C with shaking (80 rpm), and *J. lividum* and *V. paradoxus* were cultured in nutrient broth A medium (5.0 g of peptone/liter, 3.0 g of beef extract/liter, and 3.0 g of NaCl/liter) for 12 h at 25°C with shaking (80 rpm). At the end of incubation, the cell concentrations were determined microscopically. The bacterial DNA was isolated from each culture by use of a FastDNA spin kit for soil in conjunction with a FastPrep FP120 instrument.

An iCycler iQ instrument (Bio-Rad, Hercules, Calif.) was used for real-time quantitative PCRs. The species-specific primers used were as follows: a forward primer (5′-GGAAGGCATCTTTCTTGGTGGAA-3′) and a reverse primer (5′-GCCGTGTCTCAGTCCCAGTGT-3′) for *C. psychrophilum*; a forward primer (5′-ATGGCTGGAATCCCCGAGA-3′) and a reverse primer (5-TTTCGTAG CAACTAATGACAAGGGTT-3′) for *J. lividum*; and a forward primer (5′-AG GCAGCAGTGGGGAAT-3′) and a reverse primer (5′-CGTCATTAGCCCTC TTTATTAGAA-3′) for *V. paradoxus*. Primer sites were selected based on nucleotide sequences downloaded from the GenBank, DDBJ, EMBL, and RDP II databases and were designed with CLUSTAL X, v. 1.81 (National Center for Biotechnology Information, Bethesda, Md.).

Amplification was performed in a 25-μl final volume containing 1 μl of template DNA, 12.5 μl of SYBR Green PCR master mix (Applied Biosystems), and a 2.5 pM concentration of each primer. The protocol for *C. psychrophilum* and *J. lividum* included an initial denaturation step at 94°C for 10 min followed by 50 cycles of four steps (94°C for 30 s, 55°C for 30 s, 72°C for 60 s, and 78.5°C for 15 s). The protocol for *V. paradoxus* included an initial denaturation step at 94°C for 10 min followed by 50 cycles of four steps (94°C for 30 s, 60°C for 30 s, 72°C for 60 s, and 80°C for 15 s).

Fluorescence data were captured during the 72°C dwelling time. The specificity of amplification was confirmed by a melting curve analysis. Data were collected and recorded with iCycler iQ software (Bio-Rad) and were then expressed as a function of the threshold cycle, the cycle at which the fluorescence intensity in a given reaction tube rises above the background (calculated as 10 $\times$ the mean standard deviation [SD] of fluorescence in all wells over the baseline cycles). The amount of specific DNA in each sample was calculated by use of the iCycler iQ software package.

Standard curves generated with standard DNAs revealed a strong linear relationship between the log of the starting copy number and the threshold cycle.

The standard amplification curves were established with 9.71×10^3 to 9.71×10^8 cells/ml for *C. psychrophilum*, 4.16×10^2 to 4.16×10^7 cells/ml for *J. lividum*, and 1.36×10^4 to 1.36×10^7 cells/ml for *V. paradoxus* and had the following correlation coefficients: for *C. psychrophilum*, $r = 0.99$; for *J. lividum*, $r = 0.98$; and for *V. paradoxus*, $r = 0.98$.

Isolation of viable bacteria. Bacteria in the surface snow in May and July 2001 were cultured in an in situ snowmelt medium. Surface snow collected at each sampling site was melted, mixed with agar (2% [wt/vol] purified agar; Oxoid, Hampshire, United Kingdom), and boiled for more than 15 min to make agar plates. A small amount of surface snow was applied to each plate in the field and then incubated in a cold room at Tokyo Institute of Technology at 4°C for 2 weeks.

The resulting bacterial colonies were picked and suspended in sterilized water. Each suspension was heated to 96°C for 10 min to release DNA into the water. Bacterial 16S rRNA genes were obtained by PCRs with the 907F and 1407R primers as described above. The PCR products were purified by agarose gel electrophoresis and cloned into the pGEM-T vector as described above. Seven clones were randomly selected from the library for sequencing by the method described above. The nucleotide sequences obtained were compared with those in the GenBank database by use of the FASTA program and were aligned with representatives of the major bacterial divisions by the use of CLUSTAL X as described above.

Statistical analysis. Differences in the concentrations of bacteria and mineral particles among sampling seasons were examined by the Kruskal-Wallis test and Dunn's multiple comparison tests. The correlation between the concentrations of bacteria and mineral particles was examined by use of the Spearman correlation coefficient ($P < 0.05$).

RESULTS

Identification of bacterial species contained in mountain snow samples. ARDRA identified 36 clone groups. Fourteen known bacterial species were detected in the March snow samples, eight were detected in the June samples, and three were detected in the August samples (Table 1). All of the remaining species (March, 9.1%; June, 14.6%; August, 53.9%) belonged to unknown taxa.

As shown in Table 1, a broad range of bacteria were detected in the Tateyama snow samples, including soil bacteria, psychrophilic bacteria, psychrotrophic bacteria, and enteric bacteria. A large part of the 16S rRNA gene clone library was composed of organisms belonging to the β-*Proteobacteria*. Other parts of the 16S rRNA gene clone library were com-

TABLE 1. Bacteria detected in snow samples from the Tateyama Mountains

Nearest phylogenetic neighbor (GenBank accession no.)[c]	Bacterial group	Contribution (%) to whole bacterial flora (% identity) in indicated month		
		March	June	August
Corynebacterium propinquum (X81917)	*Actinobacteria*	3.0 (99.8)		
Cryobacterium psychrophilum (AJ544063)[a]	*Actinobacteria*	6.1 (98.8)	7.3 (98.8)	8.3 (98.8)
Propionibacterium acnes (AB097215)	*Actinobacteria*	22.7 (99.4)		
Tsukamurella inchonensis (AF283282)	*Actinobacteria*	3.0 (96.6)		
Enterococcus raffinosus (AJ301838)	*Bacillus*/*Clostridium* group	4.5 (99.4)		
Lactobacillus salivarius subsp. *salicinius* (AY137585)	*Bacillus*/*Clostridium* group	4.5 (99.6)		
Staphylococcus aureus (L37597)	*Bacillus*/*Clostridium* group	3.0 (99.8)		
Streptococcus mitis bv. 2 (AY005045)	*Bacillus*/*Clostridium* group	4.5 (99.8)		
Streptococcus salivarius (AY188352)	*Bacillus*/*Clostridium* group	12.1 (99.8)		
Lactobacillus salivarius subsp. *salicinius* (AY137584)	*Bacillus*/*Clostridium* group		4.2 (99.4)	
Streptococcus pneumoniae (AY281083)	*Bacillus*/*Clostridium* group	4.5 (99.6)		
Thermoanaerobacterium polysaccharolyticum (U40229)	*Bacillus*/*Clostridium* group	3.0 (79.4)	1.0 (79.4)	
Flavobacterium mizutaii (AJ438175)	*Flexibacter-Cytophaga-Bacteroides*			2.3 (92.0)
Flexibacter filiformis (AB078049)	*Flexibacter-Cytophaga-Bacteroides*			3.0 (93.2)
Flexibacter japonensis (AB078055)	*Flexibacter-Cytophaga-Bacteroides*			5.3 (93.0)
Flexibacter sp. strain MDA2495 (AY238335)	*Flexibacter-Cytophaga-Bacteroides*			1.5 (90.9)
Sphingobacterium spiritivorum (AJ459411)	*Flexibacter-Cytophaga-Bacteroides*			6.1 (92.6)
Taxeobacter ocellatus (Y18835)	*Flexibacter-Cytophaga-Bacteroides*		7.3 (95.4)	17.4 (95.0)
Rhodopseudomonas rhenobacensis (AB087719)	α-*Proteobacteria*	10.6 (98.8)		
Aquaspirillum arcticum (AB074523)	β-*Proteobacteria*		2.1 (98.2)	
Aquaspirillum autotrophicum (AB074524)	β-*Proteobacteria*		1.0 (97.0)	
Burkholderia sp. strain N3P2 (U37344)	β-*Proteobacteria*		13.5 (99.0)	
Burkholderia sp. strain SE-10 (AY568514)	β-*Proteobacteria*		4.2 (95.8)	8.3 (90.4)
Chromobacterium violaceum (AY117554)	β-*Proteobacteria*	3.0 (96.8)		
Glacier bacterium FXSI (AY315177)	β-*Proteobacteria*		1.0 (97.0)	
Janthinobacterium lividum (Y08846)[b]	β-*Proteobacteria*		34.4 (98.6)	3.0 (98.6)
Leptothrix cholodnii (X97070)	β-*Proteobacteria*			3.0 (97.2)
Rubrivivax gelatinosus (AB016167)	β-*Proteobacteria*			3.8 (97.2)
Variovorax paradoxus (AY169432)[b]	β-*Proteobacteria*		11.5 (99.0)	34.8 (99.0)
Bacterium SG-3 (AF548381)	γ-*Proteobacteria*			3.0 (85.9)
Pseudomonas sp. strain Ant9 (AF184219)	γ-*Proteobacteria*		9.4 (99.4)	
Stenotrophomonas maltophilia (AJ293464)	γ-*Proteobacteria*		3.1 (99.8)	
Escherichia coli (J01859)	γ-*Proteobacteria*	1.5 (99.8)		
Psychrobacter meningitidis (AY057116)	γ-*Proteobacteria*	9.1 (98.0)		
Rahnella sp. strain 0011 836 (AJ415576)	γ-*Proteobacteria*	3.0 (99.4)		
Terrahaemophilus aromaticivorans (AB098612)	γ-*Proteobacteria*	1.5 (99.4)		

[a] Psychrophilic bacterium detected in snow samples from all samples.
[b] Psychrotrophic bacteria detected in snow samples from both June and August.
[c] Underlining indicates species examined by real-time PCR for Fig. 2A.

posed of organisms belonging to the α-*Proteobacteria*, the γ-*Proteobacteria*, the *Flexibacter*, *Cytophaga*, *Bacteroides*, and *Bacillus*/*Clostridium* group, and the *Actinobacteria*.

C. psychrophilum, which was documented as a psychrophilic bacterium (11); *J. lividum*, which was documented as a psychrotrophic bacterium (27); and *V. paradoxus* were routinely detected in snow samples from both June 2000 and August 2000, while all other bacteria were only detected in samples collected on one specific sampling date. *J. lividum* was detected predominantly in a June snow sample (34.4% of 120 clones), whereas *V. paradoxus* was detected predominantly in an August snow sample (34.8% of 165 clones).

Bacteria isolated by culturing at a low temperature in an in situ snowmelt medium. Up to 300 bacterial colonies were isolated from the surface snow sample from May 2001, and a similar number of colonies were isolated from the surface snow sample from July 2001. These were relatively small colonies (approximately 1 to 2 mm in diameter) after 10 days of culture. Two colony morphotypes were recognized, namely, white rough and pinkish smooth colonies. Pinkish colonies were rare, whereas white colonies were predominant. The different isolated colonies represented the *Acinetobacter*, *Aquaspirillum*, *Burkholderia*, *Corynebacterium*, *Haemophilus*, *Methylobacterium*, *Propionibacterium*, *Serratia*, *Staphylococcus*, *Streptococcus*, and *Variovorax* genera.

The clone library was comprised mostly of *Variovorax* (38.2%) and *Propionibacterium* (29.4%) and, to a much smaller extent, *Staphylococcus* (5.9%) and *Streptococcus* (5.9%) sequences. The total of the remaining representatives in the library amounted to 20.6%.

Seasonal changes in specific bacterial biomass in surface snow, as estimated by real-time PCRs of 16S rRNA genes. *C. psychrophilum*, *J. lividum*, and *V. paradoxus* were subjected to biomass estimation by real-time PCR. These bacteria are documented psychrophiles or psychrotrophs or were successfully isolated by culturing at a low temperature in an in situ snowmelt medium. *V. paradoxus* was routinely detected in the snow samples by 16S rRNA gene PCR, as reported above. The biomasses (cell numbers per square centimeters) of *C. psychrophilum*, *J. lividum*, and *V. paradoxus* showed large in-

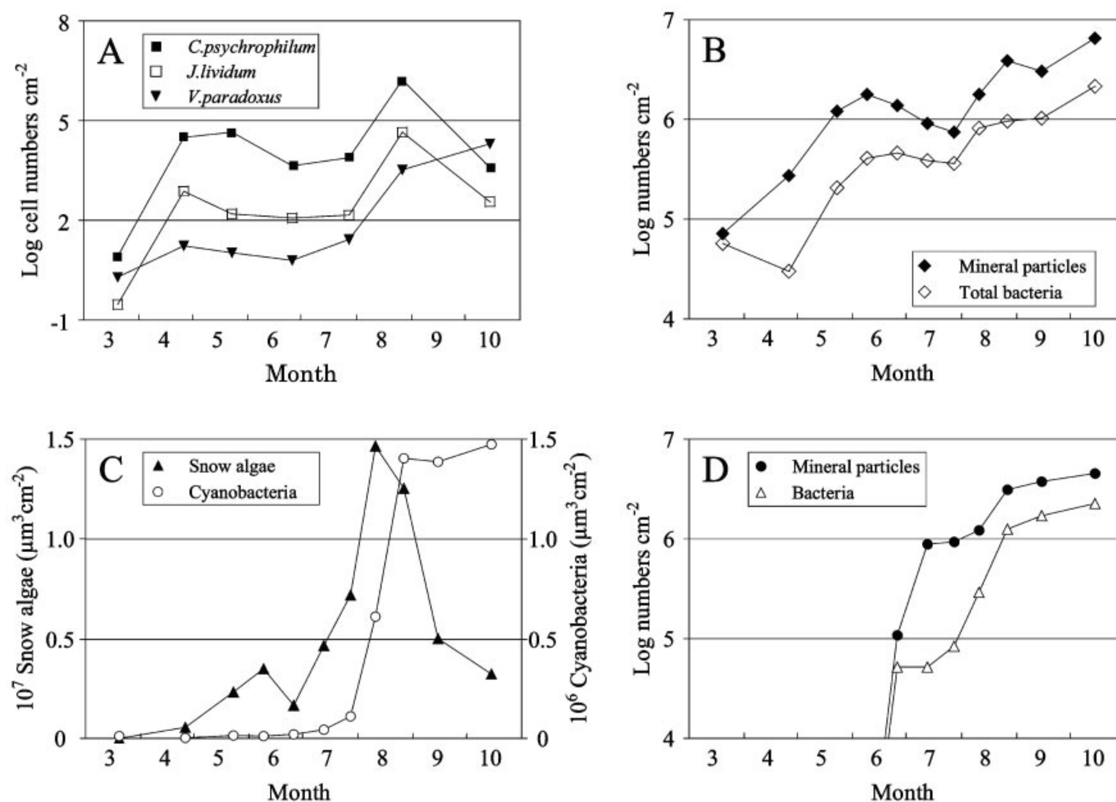

FIG. 2. Seasonal changes in bacterial biomass, algal biomass, and mineral particle density in surface snow and in numbers of bacteria and mineral particles from the atmosphere. (A) Biomasses (cell numbers per square centimeter) of *C. psychrophilum*, *J. lividum*, and *V. paradoxus* in surface snow, as estimated by real-time PCR. (B) Total bacterial biomass and numbers of small mineral particles, as estimated by microscopic counts of DAPI-stained cells and particles. (C) Biomasses of snow algae and cyanobacteria, as estimated by direct microscopic counts of DAPI-stained cells. (D) Cumulative numbers of bacteria and mineral particles supplied from the atmosphere to the snow surface, as estimated by microscopic counts of particles and DAPI-stained cells.

creases during the melting season from March to October (Fig. 2A). The biomasses of *C. psychrophilum* and *J. lividum* rapidly increased 2.0×10^5-fold and 1.5×10^5-fold, respectively, during the melting season. These biomasses increased significantly from March to April, reached a maximum in August, and dropped at the end of the melting season. In contrast, the biomass of *V. paradoxus* did not increase as rapidly during the early melting season. Until August, the quantity of the biomass of this bacterium was smaller than those of the biomasses of *C. psychrophilum* and *J. lividum*. The biomass of *V. paradoxus* continued to increase from June until October, achieving a size that was 1.0×10^4-fold larger than that in March and becoming the largest of the biomasses of the three bacteria at the end of the melting season.

Seasonal changes in total bacterial biomass and mineral particle concentration in surface snow, as estimated by direct counting. The total bacterial biomass, estimated by direct counts of DAPI-stained cells, and the count of small mineral particles with diameters of 0.5 to 3.0 μm gradually increased during the study period, especially at the end of the melting season (Fig. 2B). The decrease in the bacterial biomass and the mineral particle concentration observed from June to July was probably due to meltwater washout during midsummer. The figure shows a similar trend in total bacterial counts and mineral particle concentrations between March and October.

The seasonal changes in total bacterial biomass and mineral particle concentration were statistically significant (for bacteria, $H = 62.0$ and $P < 0.01$ by the Kruskal-Wallis test; by Dunn's multiple comparison test, $P < 0.05$; for March versus August, $P < 0.001$; and for March versus October, $P < 0.001$; for mineral particles, $H = 54.6$ and $P < 0.01$ by the Kruskal-Wallis test; by Dunn's multiple comparison test, $P < 0.05$; for March versus August, $P < 0.001$; and for March versus October, $P < 0.001$).

The extents of the increases in total bacterial counts (ca. 40-fold) and amounts of mineral particles (ca. 100-fold) from March to October were much smaller than those that were estimated by real-time PCR for *C. psychrophilum*, *J. lividum*, and *V. paradoxus*.

Although we cannot precisely compare the total biomass data estimated by the different methods, the total biomass of the three species estimated by real-time PCR made up 0.02% of the total amount of bacteria estimated by direct counts of DAPI-stained cells in March, 108.0% of that estimated in April, 22.3% of that estimated in May, 1.0% of that estimated in June, 2.1% of that estimated in July, 182.0% of that estimated in August, and 1.2% of that estimated in October. The total biomass of the three species estimated by real-time PCR exceeded the total amount of bacteria estimated by direct counts of DAPI-stained cells in April and August.

Bacteria and mineral particles supplied from the atmosphere and the snowmelt. Figure 2D shows the cumulative number of bacteria and mineral particles supplied from the atmosphere to the snow surface that would remain on the snow surface if no washout by meltwater occurred. This number increased during the study period, especially during the late melting season. The figure shows that the seasonal changes in the supply of bacteria from the atmosphere were similar to the changes in mineral particle concentrations, although the supply of mineral particles started to increase slightly earlier than that of bacteria. Both constantly increased until October. However, the increase in bacteria supplied from the atmosphere from June to August was about 20 times smaller than the increase in *C. psychrophilum* and *J. lividum*. It was also about 75-fold smaller than the increase in *V. paradoxus* from June to October.

The concentrations of bacteria in snow samples collected at various depths from a snow pit wall (total depth, 6 m) in March were 6.0×10^3 to 2.3×10^5 per ml (mean, 8.4×10^4; SD, 5.6×10^4; $n = 60$), and the corresponding concentrations of mineral particles in the same samples were 7.0×10^3 to 3.8×10^5 per ml (mean, 1.0×10^5; SD, 7.6×10^4; $n = 60$). The bacterial cell concentration and the density of mineral particles in each snow layer showed a significant, positive correlation ($R = 0.79$; $P < 0.01$), suggesting that the numbers of bacteria and mineral particles that were released to the snow surface from the snow as melting of the surface snow proceeded were also positively correlated.

These results strongly suggest that seasonal changes in the supply of bacteria from the atmosphere and the snowmelt were very similar to the changes in mineral particle concentrations.

Seasonal change in biomass of snow algae and cyanobacteria in surface snow. A unicellular algal species (*Chloromonas* sp.), a type of snow algae, and a cyanobacterial species (*Chroococcus* sp.) were observed in snow samples during the melting period. Both species increased in number during the melting period, but the growth of snow algae started earlier than that of cyanobacteria (Fig. 2C). The biomass of the snow algae increased from April to August, becoming the largest in August, and decreased at the end of the melting period. The biomass of cyanobacteria continued to increase during the late melting period from July to October. The growth of these algae and cyanobacteria started later than that of *C. psychrophilum* and *J. lividum*, which was observed from March to April.

DISCUSSION

Bacterial species identified from mountain snow samples. Considering the highly oligotrophic, aerobic, and psychrophilic nature of the snow environment, strict anaerobes or enteric bacteria were not expected to reside in the snow, even though 16S rRNA gene sequences of such bacteria were detected by cloning. These bacteria were most likely supplied by fallout from the atmosphere, as many bacteria were trapped in atmospheric dust irrespective of when the dust had been collected. The numbers of these bacteria in dust were relatively small until July (Fig. 2D). Surprisingly, enteric bacteria were detected even in March and June. Mammals and birds inhabit this region, and snow grouse (*Lagopus mutus*) and serows (*Capricornis crispus*) are the most likely sources of these bacteria because they inhabit this area in large numbers and are active throughout the year. The fact that these bacteria were detected only in a sample collected on one specific sampling date also suggests that they are occasionally supplied from the atmosphere and are not indigenous members of the snow microbiota in this region. Since we could not use an autoclave to sterilize the medium for culturing at a low temperature, it is possible that a spore-forming bacterium isolated by culturing (*Methylobacterium*) originated in heat-resistant exospores that may have been contained in the agarose powder. However, the possibility of such contamination was very small because we used a brand-new agarose powder.

Psychrophilic species such as *C. psychrophilum* and psychrotrophic species such as *J. lividum* and *V. paradoxus* are likely indigenous members of the snow microbiota in this region. *C. psychrophilum* is a chemoorganotrophic bacterium that was originally isolated from soil in Antarctica. It was reported to grow optimally at temperatures ranging from 9 to 12°C and never at temperatures higher than 18°C (11). It was also reported that *C. psychrophilum* contains a significant amount of 12-methyl pentadecanoic acid in its cellular membrane, which probably enables it to maintain membrane fluidity even at low temperatures (28). *J. lividum* was reported to be common in soil and water in temperate regions and to grow even at 2°C. This organism was reported to be chemoorganotrophic and to survive for several years in dilute peptone water at 4°C (27). *V. paradoxus* was reported to be an obligate aerobic bacterium that is common in soil and water. This species was reported to consist of two biotypes, types I and II (6, 36). Biotype I was reported to be a facultative chemoautotroph that is capable of growth by using hydrogen at a low partial pressure of oxygen (4%) but that never grows autotrophically with 20% O_2. In contrast, biotype II was reported to be a chemoorganotroph that grows well on media containing carbohydrates, organic acids, or peptone but that never grows autotrophically by using hydrogen.

The optimal temperature of *V. paradoxus* was reported to be about 30°C (6). This species was also isolated anaerobically from a Greenland glacier ice core sample recovered from a depth of about 3,042 m (25). This sample had remained at −9°C for over 100,000 years and was isolated by enrichment cultures at −2°C inoculated with melted ice.

The bacteria identified as *V. paradoxus* in this study were aerobically cultured in an in situ snowmelt medium at 4°C. Therefore, they can grow heterotrophically under cold and aerobic conditions. We cannot determine which biotype they belong to, biotype I or biotype II, because both biotypes of *V. paradoxus* can grow heterotrophically under aerobic conditions.

All three organisms, *C. psychrophilum*, *J. lividum*, and *V. paradoxus*, were aerobic heterotrophs isolated from soil and were reported to have grown at low temperatures; therefore, it is likely that these psychrophilic or psychrotrophic bacteria, which had been transported from the soil to the snow surface by the wind, grew in the cold environment of the snow during the melting season.

Bacterial growth in surface snow. The large increases in the biomasses of the three psychrophilic or psychrotrophic bacterial species, *C. psychrophilum*, *J. lividum*, and *V. paradoxus*, revealed by analyses of the surface snow samples collected

during the melting season, strongly suggested their growth in the surface snow.

The increase of bacteria in the surface snow may have been caused not only by their growth but also by other factors, including the supply of bacteria from the atmosphere, condensation by surface melting of the snow, and the effect of meltwater washout. Analyses of dust trap samples indicated that a considerable number of bacteria were supplied to the snow surface from the atmosphere during the study period. Snow samples from a 6-m-deep snow-pit wall collected before the melting season contained many bacteria. These bacteria, which were originally contained in the snow strata, should be released and condensed on the snow surface as surface melting proceeds. Seasonal changes in meltwater, which washes bacteria out of the snow surface, may also cause variations in the numbers of bacteria in the surface snow. However, it is very difficult to explain the large increases observed in the amounts of the three bacterial species by factors other than their growth in the snow. Analyses of dust trap samples and samples from the snow-pit wall suggested that seasonal changes in the supply of bacteria from the atmosphere and the snowmelt were similar to those found for mineral particles. In addition, the effect of meltwater washout on bacteria would be expected to be similar to that for bacterium-sized mineral particles because the intensity of the effect is determined primarily by particle size. Since the effects of these factors, except for growth, were considered to be similar between bacteria and mineral particles, the biomasses of these bacteria should have shown seasonal changes similar to those of mineral particles if they did not grow in the snow. However, the degrees of increase for the three species of bacteria were 100 to 4,000 times larger than that for mineral particles. In addition, the seasonal changes in the numbers of *C. psychrophilum* and *J. lividum* cells were clearly different from the changes in the numbers of mineral particles; they attained a maximum population in August which decreased thereafter, whereas the concentration of mineral particles in the surface snow continued to increase until October. Since we did not examine the species composition of bacteria supplied from the atmosphere and the snowmelt, these facts can also be explained by seasonal changes in the species composition of the bacteria supplied from the atmosphere and the snowmelt. However, it is unlikely that just these psychrophilic or psychrotrophic bacteria were supplied in such numbers at these particular times. Therefore, the data strongly suggest that these bacteria grow in the snow. In contrast to the large increases in *C. psychrophilum*, *J. lividum*, and *V. paradoxus* during the melting season (2.0×10^5-fold, 1.5×10^5-fold, and 1.0×10^4-fold increases, respectively), the total bacterial biomass increased only about 40-fold (Fig. 2B). This suggests that most of the bacteria in the surface snow belonged to species that never grew in the snow and that only a few species that adapted to the cold environment grew in the snow. This was also suggested by the similarity in the changes in total bacterial biomass and mineral particle concentration during the period of observation (Fig. 2B). However, the total bacterial biomasses estimated by direct counts of DAPI-stained cells (3.0×10^4 to 2.2×10^6 cells per cm^2) were not much larger than the total biomasses of the three species estimated by real-time PCR (1.1×10^1 to 1.8×10^6 cells per cm^2). In particular, in April and August, direct counts of DAPI-stained cells were smaller than the total biomasses of the three species estimated by real-time PCR (3.0×10^4 versus 3.2×10^4 cells per cm^2 in April and 9.6×10^5 versus 1.8×10^6 cells per cm^2 in August). This was probably due to the difference in the methods of biomass estimation.

We believe that we underestimated the total bacterial biomass by direct counts of DAPI-stained cells. Since the snow samples contained many bacterium-sized mineral particles that showed weak fluorescence by fluorescence microscopy, we only counted particles with clear fluorescence as bacteria.

The similarity in the number of bacteria recovered from the dust in the dust trap and the total number of bacteria in the surface snow (Fig. 2B and D) suggests that a major portion of the cells in the surface snow was supplied from the atmosphere with the mineral particles. A major source of the airborne bacteria seems to have been the local soil because both bacteria and mineral particles trapped by the dust trap increased during the melting season as the snow-free area increased in the vicinity of the study area. This was also suggested by the large number of soil bacterium species detected in the snow samples.

Differences in development among the bacterial species found in the surface snow suggested that their growth was promoted by different nutrients or environmental conditions in the snow. The rapid increase in *C. psychrophilum* and *J. lividum* in the early melting season from March to April, preceding the growth of snow algae and cyanobacteria, suggests that these bacterial species did not use photosynthetic products from algae and cyanobacteria for their growth during this season. They probably used dissolved organic matter contained in the meltwater. The similar evolution of these two bacterial species and the snow alga during the late melting season suggests the possibility that these bacteria grew at the expense of metabolic products from snow algae. In contrast, the growth of *V. paradoxus* at a rate similar to that of the cyanobacteria suggests some relationship between *V. paradoxus* and cyanobacteria.

Global distribution of snow-dwelling bacterial species. *C. psychrophilum* was first isolated from soil around the Syowa station at Enderby Land, Antarctica (11). This species was also reported from an Antarctic glacier, together with *J. lividum*. Christner and others (3) isolated *C. psychrophilum* and *J. lividum* from the cryoconite on the Canda Glacier in the McMurdo Dry Valley region of Antarctica by culturing them in an enrichment medium at 4 and 15°C. Cryoconite is a dark material deposited at the bottom of cylindrical melt holes (cryoconite holes) on the bare ice of glaciers. Takeuchi and others (31–33) analyzed cryoconites from glaciers of the Himalayas, Greenland, Canada, and the Arctic and reported that cryoconite is a dark-colored biomat formed by filamentous cyanobacteria and bacteria growing on the ice. They also reported that many bacteria were attached to the filamentous cyanobacteria, although the bacterial species were not identified.

V. paradoxus was isolated anaerobically from the Greenland ice core recovered from a depth of >3,000 m by the GISP2 project (25). This species was also found in two Antarctic ice core samples collected at the Yamato Range at East Queen Maud Land (25.3 to 25.5 m deep) and at Mizuho station at Enderby Land (98.5 to 99.0 m deep) by PCR and 16S rRNA gene sequencing (T. Segawa et al., unpublished data).

We also isolated *J. lividum* and *V. paradoxus* from snow

samples collected at an Alaskan glacier (Harding Icefield; 60°10′N, 149°50′W) by culturing the surface snow samples with in situ snowmelt medium at 4°C and detected all three species in surface snow samples collected at another Alaskan glacier (Gulkana glacier; 63°16′N, 145°25′W) by performing real-time PCR analyses (Segawa et al., unpublished data).

These records suggest that these three bacterial species are specialized members of the snow biota that are distributed in snow and ice environments in various parts of the world.

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