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# Microorganisms isolated from the water phase of tropospheric clouds at the Puy de Dôme: major groups and growth abilities at low temperatures

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## Keywords

Atmosphere; cloud water; microorganisms; fungi; cold environment.

## Abstract

This work constitutes the first large report on aerobic cultivable microorganisms present in cloud water. Seven cloud-event samples were collected at the Puy de Dôme summit, and cultivation was performed leading to the isolation of 71 bacterial, 42 fungal and 15 yeast strains. Most of the fungi isolated were of *Cladosporium* or *Trametes* affiliation, and yeasts were of *Cryptococcus* affiliation. Bacteria, identified on the basis of their 16S rRNA gene sequence, were found to belong to *Actinobacteria*, *Firmicutes*, *Proteobacteria* (*Alpha*, *Beta* and *Gamma* subclasses) and *Bacteroidetes* phyla, and mainly to the genera *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptomyces*, and *Arthrobacter*. These strains appear to be closely related to some bacteria described from cold environments, water (sea and freshwater), soil or vegetation. Comparison of the distribution of Gram-negative vs. Gram-positive bacteria shows that the number of Gram-negative bacteria is greater in summer than in winter. Finally, a very important result of this study concerns the ability of half of the tested strains to grow at low temperatures (5 °C): most of these are Gram-negative bacteria, and a few are even shown to be psychrophiles. On the whole, these results give a good picture of the microbial content of cloud water in terms of classification, and suggest that a large proportion of bacteria present in clouds have the capacity to be metabolically active there. This is of special interest with respect to the potential role of these microorganisms in atmospheric chemistry.

## Introduction

Clouds can be defined as atmospheric air masses in which water is condensed around particles in solid (ice crystals) or liquid form. They are low-temperature 'aquatic' environments involved in the large-scale transport of matter and energy, and so participate in the aerial connection between ecosystems, although it is still questionable whether clouds themselves can be considered as ecosystems.

It is assumed that the liquid and super-cooled water from which the free tropospheric clouds are composed provides a better temporary habitat for living airborne cells than dry air, where desiccation can be a limiting factor for growth. Thus cloud droplets may provide a medium in which these cells can divide, as suggested by Dimmick *et al.* (1979), Fuzzi *et al.* (1997) and Sattler *et al.* (2001). However, cloud water

presents some specific characteristics such as acidic pH (generally from 3 to 7), high oxidative capacity, the presence of toxic compounds such as formaldehyde, high light (including UV) exposure, and relatively low temperatures (from -15 to 10 °C at Puy de Dôme – see [http://www.obs.univ-bpclermont.fr/observ/chimie/DATA/pdd\\_Choix.html](http://www.obs.univ-bpclermont.fr/observ/chimie/DATA/pdd_Choix.html)). As a consequence, cells may require special physiological properties to remain alive in such an environment. To date, very few data are available concerning the microbial population in clouds. The reported total number of microorganisms in cloud water ranges from about 10<sup>3</sup> to 10<sup>5</sup> cells mL<sup>-1</sup> of cloud water (Sattler *et al.*, 2001; Bauer *et al.*, 2002; Amato *et al.*, 2005). Fuzzi *et al.* (1997) searched for cultivable cells in fog water at low altitude, and retrieved only three bacterial genera (*Pseudomonas*, *Bacillus* and *Acinetobacter*), and several fungi and yeasts. In tropospheric clouds, we

previously observed (Amato *et al.*, 2005) a more diversified cultivable population, consisting of many bacterial and fungal phyla. Many bacterial colonies were pigmented or spore-forming, and, as shown by their identification, some were closely related to strains isolated from cold environments. This cultivable fraction was found to represent only a small proportion of the total cells (< 1%), but our measurements of ATP concentration in cloud water support the conclusion that most of the bacteria were still alive and certainly metabolically active (unpublished data). In addition, Amato *et al.* (2005) have shown that these bacterial strains contain the enzymatic equipment necessary to transform monoacid compounds (acetate, lactate, formate) as well as formaldehyde and methanol, which are present in relatively large concentrations in cloud water or are of primary interest in atmospheric chemistry (Suzuki *et al.*, 1998; Marinoni *et al.*, 2004). Our results, together with those reported by Ariya and coworkers (Ariya *et al.*, 2002; Ariya & Amyot, 2004) in the case of diacid compounds, suggest a possible role of microorganisms in atmospheric chemistry. In order to confirm this hypothesis further, more data are needed describing the microbial population in clouds. We therefore present here the identification of culturable bacteria, yeasts and fungi isolated from seven cloud-water samples collected during different periods of the year. Growth ability at low temperatures was also investigated. This work constitutes the first large description of microbial living content to be found in cloud water.

## Material and methods

### Cloud sampling

The method and site of sampling are identical to those described in Amato *et al.* (2005). Sampling took place at the Puy de Dôme summit (1464 m a.s.l.), which is frequently covered by clouds, using a sterilized cloud-droplet impactor (Kruisz *et al.*, 1993). The Puy de Dôme is fairly isolated from the other mountains of the Dôme mountain range, and the summit is mostly covered by grass. We therefore do not expect any interaction prior to sampling between cloud droplets and vegetation. In addition, the absence of dust particles in cloud water indicates a limited contribution of local soils to the cloud composition.

### Bacteria, yeast and fungi isolation

Volumes of 0.1 mL<sup>-1</sup> of cloud water were plated directly after sampling onto three different nutrient agar media, namely Trypcase Soy (TS; Biomerieux, Marcy l'Etoile, France), used as a general medium, R2A (Reasoner & Geldreich, 1985; DIFCO, Le Pont de Claix, France) for oligotrophic microbial strain recovery, and Sabouraud (DIFCO, Le Pont de Claix, France), a medium suited to

fungal growth. Triplicates were performed for each medium at both 27 °C and 15 °C. After 3–5 days of dark aerobic incubation, each morphologically distinct colony was isolated by transplantation on the same media to obtain pure cultures.

A total of 128 microbial strains were isolated from seven cloud events, sampled between December 2003 and September 2004, including 71 bacterial, 42 fungal and 15 yeast strains.

### Growth temperature tests for bacteria

The potentialities of strains isolated as colonies to grow at 5, 17 and 27 °C were investigated. First, each bacterial strain was incubated at 5, 17 and 27 °C in one of its specific media (R2, Emerson or Trypcase Soy broths); and second, 1 mL<sup>-1</sup> was transferred into 20 mL<sup>-1</sup> of the same medium and incubated once again at 5, 17 and 27 °C. Therefore each strain was tested under different temperatures for preculture and for subsequent culture. Growth was monitored by measuring OD at 575 nm for about 120 h (5 days), and the slopes obtained during the exponential phases were used to calculate growth rates. In the case of filamentous bacteria and fungi these measurements were not possible as these organisms formed macroscopic pellets of cells during growth.

### Strain identifications

Fungi and yeasts were isolated from single colonies and their identifications were carried out using microscopic observations and physiological tests at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands). Bacteria were discriminated from eukaryotic cells (yeasts) by rapid microscopic observations and identified according to their 16S rRNA gene sequence. Cell pellets obtained after centrifugation of liquid pure cultures were resuspended in phosphate-buffered saline (PBS) solution, and their total genomic DNA was extracted using an Easy DNA Kit (Invitrogen sarl, Cergy Pontoise, France). Extracts were checked by gel electrophoresis, and 16S RNA genes were amplified by PCR. This step was carried out using universal primers for *Eubacteria*: F8-Eub (5'-AGA GTT TGA TCM TGG CTC-3') and 1492r-Univ (5'-GNT ACC TTG TTA CGA CTT-3') (Humayoun *et al.*, 2003), in which M corresponds to A or C, and N to one of the four nucleotides indifferently. About 100 ng of genomic DNA and 1.5 U of Taq Polymerase (QBiogene, Illkirch, France) were used. PCR was performed as follows: 25 cycles of 30 s at 94 °C for DNA denaturizing, 30 s at 55 °C for hybridization with primers, and 90 s at 72 °C for elongation, preceded by 5 min at 94 °C and completed by 7 min at 72 °C. PCR products were checked by gel electrophoresis, and purified on column using a Strataprep Purification Kit (Stratagene, Amsterdam,

the Netherlands). They were finally freeze-dried and sequenced by capillary electrophoresis (MWG-Biotech, Roissy CDG, France), using the previously described primer F8-Eub. Comparisons of sequences with those included in GenBank were performed with the BLASTN interface, available at <http://www.ncbi.nlm.nih.gov/BLAST/>, in order to obtain the closest neighbours and gain information about the isolation source of these neighbours.

## Results and discussion

### Description of cultivable fungi and yeasts

Table 1 gives detailed identification of the isolated yeast and fungal strains and their occurrence in our samples. Among the 42 fungi and the 15 yeasts isolated, 13 and three, respectively, were taxonomically identified. Although culturable methods present well-known limitations, a remarkable point is the wide variety of the retrieved strains, belonging to *Ascomycota*, *Basidiomycota* and *Deuteromycota* groups. Amongst *Ascomycota*, genera *Cladosporium* (four strains), *Aspergillus* (two strains, including a teleomorph

form *Eurotium*), and *Penicillium* (two strains) appear to be the most abundant. In the *Basidiomycota* phylum, seven *Polyporales* (genera *Ganoderma*, *Phanerochaeta*, *Polyporus* and four strains of *Trametes*), six *Agaricales* (one from the genus *Schizophyllum* and five strains not precisely identified) and one *Boletales* (*Coniophora* sp.) were found. Moreover, four strains belonging to the *Deuteromycota* embranchment were successfully identified (one *Trichoderma* sp. and three yeast strains: two *Cryptococcus* and one *Pseudozyma*).

All the cited genera are commonly found in natural environments, in soil and/or on vegetation. Most of them are saprophytic, growing on and causing decay of dead organic matter (plant and tree debris), or are phytopathogenic (*Botrytis cinerea*, *Fusarium avenaceum*, *Verticillium nigrescens* and *Ganoderma* sp.).

Fungal spores are frequently found in the air, and most of the genera cited here have previously been described in such environments. Genera such as *Botrytis*, *Penicillium*, *Cladosporium* and *Aspergillus* have been retrieved from indoor dry-air samples (Lin et al., 1999; Radon et al., 2002). *Aspergillus* and *Cladosporium* are certainly the genera most described in microbial studies of the atmosphere: in addition to their

**Table 1.** Isolated fungal and yeast strains, and respective main isolation sources and characteristics

Strain	Phylum	Common isolation sources and main characteristics	Number of isolates
<b>Fungi</b>			
<i>Acremonium</i> sp.	<i>Ascomycota</i>	Soil, plant debris; saprophytic	1
<i>Aspergillus fumigatus</i>	<i>Ascomycota</i>	Air, soil, decaying organic matter; saprophytic	1
<i>Botrytis cinerea</i>	<i>Ascomycota</i>	Plants; phytopathogenic	1
<i>Cladosporium</i> spp.	<i>Ascomycota</i>	Wide spread, soil, plants; saprophytic	4
<i>Coniophora</i> sp.	<i>Basidiomycota</i>	Wood; saprophytic	1
<i>Eurotium rubrum</i>	<i>Ascomycota</i>	Soil; saprophytic; Teleomorph form of <i>Aspergillus rubrobrunneus</i>	1
<i>Fusarium avenaceum</i>	<i>Ascomycota</i>	Plants; phytopathogenic	1
<i>Ganoderma</i> sp.	<i>Basidiomycota</i>	Trunks; intense sporing; parasitic and saprophytic	1
<i>Penicillium</i> cf. <i>sclerotiorum</i>	<i>Ascomycota</i>	Soil; saprophytic	1
<i>Penicillium thomii</i>	<i>Ascomycota</i>	Wide spread; sea water, plants, soil; saprophytic	1
<i>Phanerochaeta</i> sp.	<i>Basidiomycota</i>	Wood; saprophytic	1
<i>Plectosphaerella cucumerina</i>	<i>Ascomycota</i>	Soil, decaying vegetation; saprophytic; teleomorph from of <i>Plectosporium tabacinum</i>	1
<i>Podospora</i> sp.	<i>Ascomycota</i>	Herbivore dung; coprophilous	1
<i>Polyporus tuberaster</i>	<i>Basidiomycota</i>	Decaying wood; saprophytic	1
<i>Schizophyllum</i> sp.	<i>Basidiomycota</i>	Dead wood; saprophytic	1
<i>Trametes</i> spp.	<i>Basidiomycota</i>	Stumps, decaying wood; saprophytic	4
<i>Trichoderma citrinoviride</i>	<i>Deuteromycota</i>	Decaying wood and vegetation; saprophytic	1
<i>Verticillium nigrescens</i>	<i>Ascomycota</i>	Plant; phytopathogenic	1
<i>Agaricales</i> indet.	<i>Basidiomycota</i>	–	5
ND	–	–	13
	Total		42
<b>Yeasts</b>			
<i>Cryptococcus albidus</i>	<i>Deuteromycota</i>	Soil, water, leaves, air; saprophytic	1
<i>Cryptococcus laurentii</i>	<i>Deuteromycota</i>	Water, plants; saprophytic	1
<i>Pseudozyma</i> sp.	<i>Deuteromycota</i>	Plant materials; parasitic	1
ND	–	–	12
	Total		15

ND, Not determined.

being detected indoors, they are widespread in outdoor air (Fulton, 1966b; Baxter & Cookson, 1983; Katial *et al.*, 1997; Durand *et al.*, 2002; Wittmaack *et al.*, 2005), and also in nonprecipitating atmospheric water in the case of *Aspergillus* (Fuzzi *et al.*, 1997; Amato *et al.*, 2005). Concerning *Cladosporium* spp., not yet described in cloud water, their presence in our samples is not surprising in view of the number of studies reporting their presence in the air. Because *in situ* contamination can be excluded, it appears that, as for the other identified genera, *Cladosporium* spp. are integrated into droplets during cloud formation, meaning that such fungal spores can reach quite high altitudes despite their size, and can be transported over long dis-

tances. This remark also holds true for *Trichoderma* and *Fusarium* genera, collected at high altitude by Fulton (1966a), and also found in our cloud samples. Concerning yeasts, *Cryptococcus* members have been retrieved in alpine habitats, where they appeared as cold-adapted microorganisms (Bergauer *et al.*, 2005).

### Description of cultivable bacterial population

Table 2 presents a detailed view of bacterial genera cultured from each sample. The majority of bacterial isolates (61 from the 71 isolates) were successfully identified by sequencing their 16S rRNA gene and making a comparison with

**Table 2.** Detailed bacterial strain isolation for each cloud event sampled

Phylum	Genus	12/12/ 2003	01/15/ 2004	01/21/ 2004	02/16/ 2004	06/24/ 2004	07/08/ 2004	09/23/ 2004	Number of isolated strains
Actinobacteria	<i>Streptomyces</i>		1		1	1		2	5
	<i>Arthrobacter</i>	1	2		1				4
	<i>Micrococcus</i>	1			1	1			3
	<i>Saccharothrix</i>			1					1
	<i>Kocuria</i>				1				1
	<i>Agromyces</i>	1							1
	<i>Cellulomonas</i>	1							1
	<i>Tetrasphaera</i>	1							1
	<i>Leucobacter</i>	1							1
	<i>Luteococcus</i>		1						1
	<i>Agrococcus</i>				1				1
	<i>Curtobacterium</i>						1		1
	<i>Frigoribacterium</i>							1	1
	<i>Nocardioides</i>				1				1
Uncertain genus*	1					1			2
Total		7	4	1	6	3	1	3	25
Firmicutes	<i>Staphylococcus</i>	2	4	1					7
	<i>Bacillus</i>	1	1		1				3
	<i>Paenibacillus</i>				1				1
	Unidentified Bacilliales*					1			1
	Total	3	5	1	2	1	0	0	12
Bacteroidetes	<i>Pedobacter</i>			1					2
	<i>Flavobacterium</i>							1	1
	<i>Sphingobacterium</i>							1	1
	Total	0	0	1	0	0	0	2	3
Alphaproteobacteria	<i>Sphingomonas</i> sp.	2			2			2	6
	<i>Methylobacterium</i>				1				1
	<i>Aurantimonas</i>				1				1
	Total	2	0	0	4	0	0	2	8
Betaproteobacteria	<i>Massilia</i>	1							1
	<i>Zoogloea</i>	1							1
	Total	2	0	0	0	0	0	0	2
Gammaproteobacteria	<i>Pseudomonas</i>	1		1		1	2	3	8
	<i>Moraxella</i>		2						2
	<i>Pantoea</i>					1			1
	Total	1	2	1	0	2	2	3	11
Not determined		4	5		1				10
	Total	19	16	4	13	6	3	10	71

\*Genus not accessible with sequenced fragment length.

sequences in GenBank. Partial sequences have been lodged in GenBank, and accession numbers are specified in Tables 3 and 4.

### General overview

Although only culturable bacteria were identified from the cloud-water samples, and not the total bacterial population, a large variety distributed among at least 28 genera can be described. Among the 61 isolates identified, 37 Gram-positive (61%) (25 *Actinobacteria* and 12 *Firmicutes*) and 24 Gram-negative (39%) (three *Bacteroidetes*, eight *Alphaproteobacteria*, two *Betaproteobacteria* and 11 *Gammaproteobacteria*) strains were found. The predominance of Gram-positive strains cultured from cloud water is consistent with previous works, showing that the Gram-positive cultivable fraction is generally more abundant in air than the Gram-negative one. Shaffer & Lighthart (1997) found about 80% of the cultivable population to be affiliated to Gram-positive phyla in dry air. This is supported by laboratory experiments showing that Gram-negative bacteria lose cultivability after being aerosolized (Heidelberg et al., 1997).

*Actinobacteria* was the most important phylum in terms of number of isolates and of genera represented: we found this affiliation for about half of the total number of genera identified. Genera *Streptomyces* (four strains), *Arthrobacter* (four strains) and *Micrococcus* (two strains) were the dominant ones, others (at least 11 different genera) being represented by only a single strain. The other phylum of Gram-positive bacteria (*Firmicutes*) was also highly represented, with respectively five and three distinct strains of the genera *Staphylococcus* and *Bacillus*.

Although the Gram-positive population is spread evenly amongst a great number of genera, the less-well represented Gram-negative group includes a few genera that are particularly dominant, namely *Pseudomonas* (*Gammaproteobacteria*) and *Sphingomonas* (*Alphaproteobacteria*), with respectively seven and five distinct strains isolated. These two genera are found to be the most abundant ones amongst all isolates. Only a few *Betaproteobacteria* and *Bacteroidetes* (also called *Cytophaga-Flavobacterium-Bacteroides*, CFB-group) were cultured from the samples, with respectively only two and three strains from these phyla.

Shaffer & Lighthart (1997) retrieved especially the genera *Bacillus* and *Curtobacterium* in their air samples. They also cited *Arthrobacter* and *Micrococcus* as being present. As in our observations of cloud droplets, *Pseudomonas* was the most abundant Gram-negative genus that they recovered from air samples. In another study focusing on airborne microbial content in an urban environment, cultivable *Staphylococcus* was found to be the predominant genus (Mancinelli & Shulls, 1978). *Staphylococcus* genus is also well represented in one of our samples, but back-trajectory

plotting and chemical analysis (not shown) did not reveal any special urban influence on its composition. There is very little literature concerning the microbial content of cloud water; however, in fog, *Bacillus* and *Pseudomonas* seem to be the most commonly cultivated genera (Fuzzi et al., 1997). In a previous work based on only two cloud events at the Puy de Dôme (Amato et al., 2005), we also isolated *Pseudomonas* and *Bacillus* species, in addition to other genera such as *Streptomyces*, *Micrococcus*, *Microbacterium*, *Flavobacterium*, *Kocuria*, *Arthrobacter*, and *Clavibacter*.

### Detailed identification of bacteria

Blast results obtained from the 16S rRNA gene sequences of our Gram-positive and Gram-negative isolates are respectively shown in Table 3 and 4. All sequences matched with known ones by a value at least as high as 97%, except for one that reached 96% (5b-7). Strains closely related to our isolates from cloud water were obviously from various origins, which can be regrouped as follows: aquatic environments (surface and ground water), extreme cold environments (polar and glacier samples, frozen soils), soil (sediment and other soils), vegetation (grass and plant leaves), and fauna (insects and other animals). A few strains cannot be included in these groups: strains 3b-4 (referenced as undetermined *Actinobacterium*) and 3b-20 (*Leucobacter aridicollis*) are *Actinobacteria* closely related to isolates from chromium- and nuclear-contaminated environments, while 3b-6 (*Bacillus simplex*) and 3b-19 (*Massilia* sp.) are neighbours to isolates recovered from mural painting and cleanrooms. In eight cases, the isolation source was not available.

The Gram-positive group appears to have a wide range of origins. Here we found highly diversified neighbour strains, from human-blood isolates (5b-7, *Luteococcus sanguinus*), to ancient Siberian permafrost (5b-15, *Arthrobacter* sp.) and sugar beet (13b-4, *Curtobacterium flaccumfaciens*). This suggests that, rather than being limited to carrying strains of environmental origin, clouds can also carry some potentially human pathogenic germs alive. Interestingly, many of the Gram-positive strains are close to isolates from zones such as deep-sea or coastal sediments, or groundwater. This particularly concerns the genera *Micrococcus*, *Staphylococcus*, *Streptomyces* and *Arthrobacter*. Genera such as *Micrococcus*, *Bacillus* and *Staphylococcus* are widespread in a large variety of environments, and are also present in cloud water.

*Alphaproteobacteria* isolates mainly belong to the genus *Sphingomonas*, but also to *Methylobacterium* (7b-5) and *Aurantimonas* (7b-14). All of them are closely related to microorganisms found in cold environments, and this group is broadly more often isolated from extreme cold environments (Christner et al., 2001; Zhang et al. 2002; Foght et al., 2004). Their physiological properties certainly make them fitted to living in an environment comparable to

Table 3. Gram-positive isolates

Phylum	Strain	Seq. length (bp)	A.N	Date of sampling	Closest related neighbour	A.N	% homology (bp)	isolation source	Growth rate (OD/h)			
									at 5 °C	at 17 °C	at 27 °C	at 27 °C
<i>Actinobacteria</i>	3b-2	869	DQ512738	12/12/2003	<i>Micrococcus luteus</i> isolate CV39	AJ717368	99% (869/872)	Alkaline groundwater	+	+	+	+
	3b-4	860	DQ512739	12/12/2003	<i>Actinobacterium RG-9</i>	AY561575	99% (852/860)	High-Level Nuclear Waste-Contaminated	+	+	+	+
	3b-5	679	DQ512740	12/12/2003	<i>Agromyces neolithicus</i> strain 23-23	AY507128	98% (642/652)	Not available	+	+	+	+
	3b-7	836	DQ512742	12/12/2003	<i>Arthrobacter rhombi</i> strain F98_3HR69	Y15885	99% (828/836)	Greenland halibut	+	+	+	+
	3b-8	510	DQ512743	12/12/2003	<i>Cellulomonas</i> sp. 73NP9	AB242681	99% (507/510)	Tomato leaf	+	+	+	+
	3b-18	790	DQ512754	12/12/2003	<i>Tetrasphaera</i> sp. Ellin150	AF408992	98% (777/792)	Pasture soil	+	+	+	+
	3b-20	848	DQ512748	12/12/2003	<i>Leucobacter aridicollis</i> type strain L9	AJ781047	99% (842/847)	Chromium contaminated environment	+	+	+	+
	5b-4	698	DQ512755	01/15/2004	<i>Streptomyces albidoflavus</i>	AJ002090	99% (698/699)	Not available	+	+	+	+
	5b-7	652	DQ512756	01/15/2004	<i>Luteococcus sanguinus</i> strain CCUG 33897T	AJ416758	96% (599/619)	Human blood	+	+	+	+
	5b-11	566	DQ512758	01/15/2004	<i>Arthrobacter</i> sp. isolate An29	AJ551167	100% (566/566)	Deep sea sediment	+	+	+	+
	5b-15	545	DQ512760	01/15/2004	<i>Arthrobacter</i> sp. 255-8a	AY444852	99% (542/545)	Ancient Siberian permafrost	+	+	+	+
	6b-3	805	DQ512765	01/21/2004	<i>Saccharothrix tangerinus</i> strain:MK27-91F2	AB020031	98% (793/806)	Not available	+	+	+	+
	7b-1	779	DQ512767	02/16/2004	<i>Streptomyces ciscaucasicus</i> strain DSM 40275	AY508512	99% (774/781)	Not available	+	+	+	+
	7b-2	766	DQ512768	02/16/2004	<i>Agrococcus jenensis</i>	X92492	100% (766/766)	Frozen compost soil	+	+	+	+
	7b-4	605	DQ512769	02/16/2004	<i>Micrococcus luteus</i> CV31	AJ717367	99% (903/905)	Alkaline groundwater	+	+	+	+
	7b-7	659	DQ512772	02/16/2004	Rhizosphere soil bacterium isolate RSI-25 ( <i>Nocardioles</i> sp.)	AJ252592	99% (658/659)	Rhizosphere soil	+	+	+	+
	7b-9	647	DQ512773	02/16/2004	<i>Arthrobacter oxydans</i>	X83408	99% (646/648)	Not available	+	+	+	+
	7b-12	529	DQ512775	02/16/2004	<i>Kocuria rhizophila</i>	Y16264	99% (528/529)	Cattail of swamp plant	+	+	+	+
	12b-4	398	DQ512779	06/24/2004	Uncultured bacterium clone C13_D15	AY991105	98% (395/399)	Mouse caecum	+	+	+	+
	12b-6	777	DQ512781	06/24/2004	<i>Micrococcus luteus</i> CV31	AJ717367	100% (777/777)	Alkaline groundwater	+	+	+	+

Table 3. Continued.

Phylum	Strain	Seq. length (bp)	A.N	Date of sampling	Closest related neighbour	A.N	% homology (bp)	Isolation source	Growth rate (OD/h)		
									at 5 °C	at 17 °C	at 27 °C
	12b-10994	DQ512784	DQ512784	06/24/2004	<i>Streptomyces albidoflavus</i>	AJ002090	99% (991/994)	Not available	–	–	–
	13b-4	DQ512787	DQ512787	07/08/2004	<i>Curvobacterium flaccumfaciens</i> pv. <i>Beticola</i>	AY273208	100% (934/934)	Sugar beet	–	+	++
	14b-9	DQ512793	DQ512793	09/23/2004	<i>Streptomyces</i> sp. N0143	AY754724	100% (470/470)	Coastal sediment	–	–	–
	14b-12982	DQ512795	DQ512795	09/23/2004	<i>Streptomyces</i> sp. N0130	AY754722	99% (980/984)	Coastal sediment	–	–	–
	14b-13427	DQ512796	DQ512796	09/23/2004	<i>Frigoribacterium</i> sp. G1C6	AY439262	99% (418/422)	Glacial ice core from Greenland	–	++	+
<i>Firmicutes</i>	3b-6	DQ512741	DQ512741	12/12/2003	<i>Bacillus simplex</i> strain LMG 21002A1316308	AY462205	100% (1397/1397)	Mural painting environment	–	++	++++
	3b-15	DQ512752	DQ512752	12/12/2003	<i>Staphylococcus epidermidis</i> isolate CV64	AJ171377	99% (950/952)	Alkaline groundwater	–	–	–
	3b-16	DQ512753	DQ512753	12/12/2003	Bacterium G24 ( <i>staphylococcus</i> sp.)	AY345395	99% (936/941)	Green Lake water	–	–	–
	5b-1	DQ512749	DQ512749	01/15/2004	<i>Bacillus pumilus</i> strain DF20	AY462205	99% (896/898)	Not available	–	–	–
	5b-3	DQ512750	DQ512750	01/15/2004	Bacterium G24 ( <i>staphylococcus</i> sp.)	AY345395	100% (896/896)	Green Lake water	++	++	++
	5b-6	DQ512751	DQ512751	01/15/2004	<i>Staphylococcus cohnii</i> isolate CV38	AJ171378	100% (889/889)	Alkaline groundwater	++++	++++	++++
	5b-8	DQ512757	DQ512757	01/15/2004	Uncultured <i>Staphylococcus</i> sp. clone: MgMJD-019	AB234514	99% (897/898)	Gut of a fungus-growing termite	–	++++	++
	5b-16	DQ512761	DQ512761	01/15/2004	<i>Staphylococcus equorum</i> strain JH6	DQ232735	100% (722/722)	Poultry farm	++	+++	+++
	6b-1	DQ512763	DQ512763	01/21/2004	Bacterium G24 ( <i>staphylococcus</i> sp.)	AY345395	99% (961/962)	Green Lake water	+	++	++++
	7b-6	DQ512771	DQ512771	02/16/2004	<i>Paenibacillus</i> sp. isolate Eimt3	AM062705	100% (667/667)	Lichen	+	+	+
	7b-11	DQ512774	DQ512774	02/16/2004	<i>Bacillus licheniformis</i> strain HK-1	AY530960	99% (847/850)	Poultry waste	+	+	+
	12b-7	DQ512782	DQ512782	06/24/2004	Bacillales bacterium s50-7-u8f	DQ305315	99% (750/755)	Rice field soil	–	–	+

Genera and species names refer to the closest neighbours established through a BLAST search. Growth rates observed at 5 °C, 17 °C and 27 °C are indicated where established. <sup>i</sup>Growth rates (OD/h) during the exponential phase of growth) are –, < 0.02; +, 0.02–0.049; ++, 0.05–0.29; +++, 0.3–0.59; +++++, > 0.6.

A.N, accession number in GenBank.



Table 4. Gram-negative isolates

Phylum	Strain	Seq. length (bp)	A.N	Date of sampling	Closest related neighbour	A.N	% homology (bp)	Isolation source	Growth rate (OD/h)			
									at 5 °C	at 17 °C	at 27 °C	
Bacteroidetes	6b-2	804	DQ512764	01/21/2004	<i>Pedobacter</i> sp. type strain HHS22	AJ583425	99% (792/800)	Himalayan glacier				
	14b-7	898	DQ512791	09/23/2004	<i>Flavobacterium</i> sp. EP101	AF493649	98% (862/873)	River taff epilithon	+	-	-	
	14b-8	768	DQ512792	09/23/2004	Uncultured <i>Sphingobacterium</i> sp. clone TM15_56	DQ279367	97% (733/748)	Truffle	-	+	+	
	Alphaproteobacteria	3b-10	1007	DQ512744	12/12/2003	<i>Sphingomonas</i> sp. Pmxh3	DQ314734	99% (1004/1007)	Subnival plant			
		3b-11	609	DQ512745	12/12/2003	<i>Sphingomonas</i> sp. TSBY-61	DQ166168	99% (608/609)	Frozen soil			
		7b-5	682	DQ512770	02/16/2004	<i>Methylobacterium</i> sp. strain: PB73AB220078		100% (682/682)	Terrestrial freshwater			
		7b-13	784	DQ512776	02/16/2004	<i>Sphingomonas</i> sp. M3C2038-B	AF395031	99% (784/785)	Antarctic subglacial lake Vostok	+	++	++
		7b-14	703	DQ512777	02/16/2004	Endophytic bacterium Enf15 (closely related to <i>Aurantimonas</i> sp.)	DQ339602	100% (703/703)	Alpine subnival plants	-	++	++
		7b-15	858	DQ512778	02/16/2004	Glacier bacterium FXS25 ( <i>Sphingomonas</i> sp.)	AY315166	97% (841/859)	New Zealand subglacial sediments	++	+++	+++
		14b-5	950	DQ512789	09/23/2004	<i>Sphingomonas</i> sp. M3C2038-B	AF395031	99% (950/952)	Antarctic subglacial lake Vostok			
		14b-6	968	DQ512790	09/23/2004	<i>Sphingomonas</i> sp. isolate J05	AJ864842	99% (960/964)	High mountain lake water	-	+	-
	Betaproteobacteria	3b-12	1000	DQ512746	12/12/2003	<i>Zoogloea ramigera</i> ATCC 25935	X74914	99% (921/930)	Trickling filter of a sewage plant	++	+++	++
		3b-19	869	DQ512747	12/12/2003	Uncultured <i>Massilia</i> sp. clone KL-11-1-5	AF408326	98% (860/869)	Clean-room facility			
	Gammaproteobacteria	3b-1	858	DQ512737	12/12/2003	<i>Pseudomonas syringae</i> pv. <i>corlyi</i> strain NCPPB 4273	AJ889841	99% (852/854)	Hazelnut			
5b-14		815	DQ512759	01/15/2004	<i>Moraxella osloensis</i>	AY043376	99% (814/815)	Respiratory secretions				
5b-17		808	DQ512762	01/15/2004	<i>Moraxella phenylpyruvica</i>	AF005192	99% (791/792)	Spinal fluid				
6b-4		813	DQ512766	01/21/2004	<i>Pseudomonas rhizosphaerae</i>	AY152673	98% (799/813)	Rhizospheric soil of grasses	+	++	+	
12b-5		655	DQ512780	06/24/2004	<i>Pantoea agglomerans</i>	AY395012	99% (652/656)	Moth larval midgut		+++	+++	
12b-8		719	DQ512783	06/24/2004	<i>Pseudomonas syringae</i> pv. <i>atropurpurea</i>	AB001440	99% (718/720)	Gramineaceous grass	+	+++	+++	

Table 4. Continued.

Phylum	Strain	Seq. length (bp)	A.N	Date of sampling	Closest related neighbour	A.N	% homology (bp)	Growth rate (OD/h)			
								Isolation source	at 5 °C	at 17 °C	at 27 °C
	13b-2	887	DQ512785	07/08/2004	<i>Pseudomonas syringae</i> pv. atropurpurea	AB001440	100% (887)	Graminaceous grass			
	13b-3	1004	DQ512786	07/08/2004	<i>Pseudomonas graminis</i>	Y11150	99% (1003/1005)	Phyllosphere of grasses	+	+	++
	14b-2	898	DQ512788	09/23/2004	<i>Pseudomonas</i> sp. SE22#1a	AY263477	99% (896/898)	Alpine soil	+	++	++
	14b-10	780	DQ512794	09/23/2004	<i>Pseudomonas</i> sp. PH20A	AY835585	100% (780/780)	Leaf spots of bean			
	14b-14	854	DQ512797	09/23/2004	<i>Pseudomonas viridiflava</i> strain RMX23.1a	AY574912	100% (854/854)	<i>Arabidopsis thaliana</i>	+	+	-

Genera and species names refer to the closest neighbours established through a BLAST search. Growth rates observed at 5 °C, 17 °C and 27 °C are indicated where established. Growth rates (OD/h during the exponential phase of growth) are -, < 0.02; +, 0.02–0.049; ++, 0.05–0.29; +++, 0.3–0.59; +++++, > 0.6. A.N, accession number in GenBank.

cloud water, as the low nutrient levels and cold conditions improve their survival rate. *Gammaproteobacteria* are for their part largely represented by *Pseudomonas* spp. (eight isolates out of 11), with the additional presence of two *Moraxella* spp. strains and one *Pantoea agglomerans*. Vegetation appears to be the major source of *Pseudomonas*, but this genus is known to be very ubiquitous and its presence was not surprising here. Furthermore, *Pseudomonas* have even been recovered from environments such as old glacial ice (about 750 000 years old) (Christner et al., 2003), testifying to their high resistance to extreme conditions. The strains from our cloud samples were often phytopathogenic, such as the example of *P. syringae* (3b-1, 12b-8 and 13b-2) (Sawada et al., 1997; Scortichini et al., 2002). For such strains, air constitutes an important way of dissemination, and their occurrence in our samples was to be expected. To retrieve *P. syringae* in cloud water is also of interest regarding the role that such a bacterium could play in the physics and chemistry of clouds. First, this species was found in Antarctic samples and is capable of cold adaptation (Seshu Kumar et al., 2002). Second, it has been demonstrated to have a very efficient ice-nuclei-forming activity (Cochet & Widehem, 2000), signifying its potential ability to initiate ice-crystal formation in supercooled clouds at relatively high temperatures. In the past, Schnell & Vali (1972) and Lindemann et al. (1982) suspected ice-nuclei to be produced in relation to vegetation, but *P. syringae* had not then been described in cloud samples. In addition, other species found in our samples are known to have a substantial ice-nuclei activity, for example bacteria of *Pantoea* affiliation and the fungi *Fusarium avenaceum* (Pouleur et al., 1992). We provide here proof of their regular presence in cloud droplets at different periods of the year, and it is therefore likely that such microorganisms are involved in microphysical processes occurring in clouds.

Other *Gammaproteobacteria* isolates (*Moraxella*) are more related to fauna emissions. The two last phyla of Gram-negative bacteria that were detected, *Betaproteobacteria* and *Bacteroidetes*, were more sporadic, but the presence of the *Pedobacter* sp. (6b-2), very similar to an isolate from a Himalayan glacier, can be noted (Shivaji et al., 2005).

Five bacterial strains were found several times in different samples. These were two *Actinobacteria* species [*Streptomyces albidoflavus* from a nonspecified source (5b-4 and 12b-10), and *Micrococcus luteus* CV31 from alkaline groundwater (7b-4 and 12b-6)], one *Firmicutes* [*Bacterium* G24, which is of *Staphylococcus* affiliation (3b-16, 5b-3 and 6b-1)], one *Gammaproteobacteria* [*Sphingomonas* sp. M3C203B-B from the Antarctic subglacial lake Vostok (7b-13 and 14b-5)], and one *Gammaproteobacteria* [*Pseudomonas syringae* pv. atropurpurea from graminaceous plant (12b-8 and 13b-2)].

### Variations of bacterial composition with season

The results presented in Table 2 show that the total number of cultivable bacteria varies from one cloud event to another. In this section we briefly discuss the influence of the season on these variations. During summer, the proportion of Gram-positive cultivable bacteria (42.1% of the isolates) was less than that of Gram-negative ones (57.9% of the isolates). From summer to winter, the relative proportion of each group (Gram-positive and Gram-negative) changed, and the Gram-negative group became dominant.

In snow samples collected at an altitude of about 2500 m in Japan, Segawa *et al.* (2005) observed that the Gram-positive population contributed 71% of the whole bacterial flora in March, whereas it represented not more than 13% in June, and decreased to 8% in August. This is quite similar to what was observed in our cloud samples.

A first possible reason explaining the decrease of Gram-positive isolates in summer could be their greater sensitivity to the damaging effects of UV, more important in summer, as compared with Gram-negative ones. Recently, Aguogué *et al.* (2005) showed that *Gammaproteobacteria* (Gram-negative) was the more represented group among the highly resistant to solar radiation bacteria isolated from the surface of the sea. A second reason could very possibly be the influence of summer on the preferential occurrence of microbial strains on vegetation which is far more developed in summer than in winter. Figure 1 gives a more detailed bacterial composition for the two periods, providing averages of the occurrence of each phylum per sample, and confirms our two previous hypotheses. First, it shows that the observed difference between winter and summer is in great part the result of a decrease in the *Firmicutes* proportion from 21% to 5%, combined with an increase in *Gammaproteobacteria* from 8% to 37%. Such a fact can be supported by the higher resistance of *Gammaproteobacteria* to solar radiation, as previously mentioned. Second, the

development of vegetation in summer could also explain the increase of this phylum, which includes the *Pseudomonas* genus, well known to grow on plants.

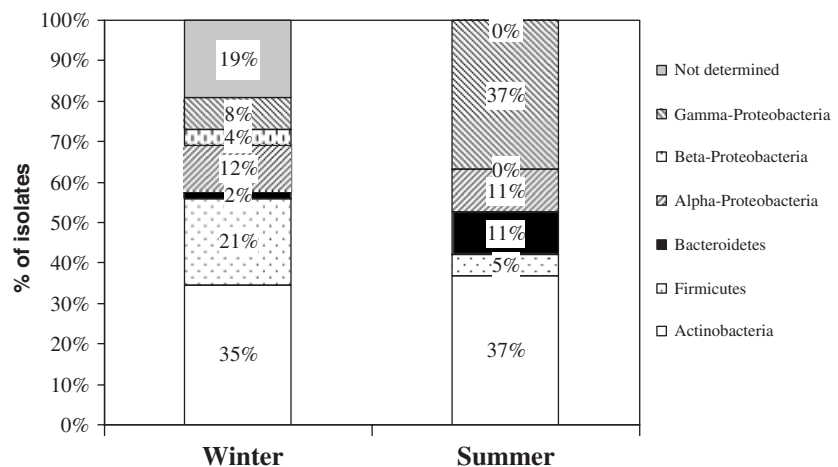
### Abilities to grow at low temperatures

Some isolated strains were tested for their aerobic growth at 5, 17 and 27 °C. The results are presented in Tables 3 and 4. Atmospheric temperature is recorded continuously at puy de Dôme (VAISALA PT 100 sensor), showing an average value of  $5.2 \pm 1.2$  °C. The lowest incubation temperature was therefore chosen in the range of this mean annual temperature. It is also interesting to note that winter-time temperatures at puy de Dôme regularly go down to  $-15$  °C. Twenty strains were tested for the three temperatures, and for eleven others only the 17 and 27 °C tests could be performed because of technical problems. For convenience, we created four classes of growth ability according to the growth rates expressed as OD values per hour (see legends of Tables 3 and 4).

For two strains (7b-7 *Nocardioides* sp. and 5b-1 *Bacillus pumilus*), the growth rates remained very low whatever the temperature investigated.

Six strains out of 38 tested (about 16%) grew faster at 27 °C than at 5 and 17 °C. All of them are of Gram-positive affiliation: they include four *Actinobacteria* and two *Firmicutes* strains. Some strains are of special interest because they grow better at 17 °C: 14b13 (*Frigobacterium* sp.), 5b8 (*Staphylococcus* sp.), 6b4 (*Pseudomonas rhizosphaerae*), and 3b12 (*Zoogloea ramigera*). The two last strains also grow at 5 and 27 °C, and can thus be considered as psychrotolerant.

More interesting for this study of clouds are those strains able to grow at a low temperature close to the natural atmospheric environment, i.e. 5 °C. This was the case for 11 strains out of 20 tested (about 55%), including five *Gammaproteobacteria* (*Pseudomonas* spp.), two *Firmicutes* (*Paenibacillus* sp., and *Staphylococcus* sp.), two



**Fig. 1.** Global composition in aerobic cultivable bacteria during winter and summer. The contribution of each phylum to an averaged cloud-water sample is given with respect to the season.

*Alphaproteobacteria* (*Sphingomonas* spp.), one *Betaproteobacteria* (*Zoogloea ramigera*), and one *Bacteroidetes* (*Flavobacterium* sp.): the large majority are Gram-negative strains. It can be noted that three strains have a relatively high growth rate at 5 °C: 3b12 (*Zoogloea ramigera*), 5b16 (*Staphylococcus equorum*), and 7b15 (*Sphingomonas* sp.). Finally, two strains are also remarkable because they grow at 5 °C but not at 17 and 27 °C, and can therefore be considered as true psychrophiles: 14b14 (*Pseudomonas viridiflava*) and 14b7 (*Flavobacterium* sp.), according to the definition of Morita (1975).

Clearly, Gram-negative strains are much more efficient at growing at low temperatures, because 75% (9/12) of those tested have this capacity. Among the Gram-positive strains, only 25% (2/8) presented this ability. This is consistent with observations made on iced environments such as the poles and high mountains, where the Gram-negative community is dominant (Alfreider *et al.*, 1996; Brinkmeyer *et al.*, 2003; Groudieva *et al.*, 2004). These results are also comparable with those obtained by Groudieva *et al.* (2004), who found only a few truly psychrophilic strains among a population dominated by psychrotolerants in a polar environment. In addition, many colonies we isolated were yellow to orange pigmented (not shown), as is often reported for bacteria cultivated from cold environments (Fong *et al.*, 2001; Foght *et al.*, 2004).

## Conclusion

This work is the first to report an extensive study of aerobic cultivable microorganisms present in atmospheric water. This water was collected at the top of the Puy de Dôme mountain, over a period of more than a year and a half corresponding to seven cloud events. Although culture methods do not allow all members of the population to be described, they give very valuable information about the physiology of microorganisms that are really alive in clouds. This is of special interest given that the cloud medium can prevent microorganisms from surviving because of the harsh conditions such as cold temperature, UV exposure, low pH, and oxidative environment.

General considerations can be drawn concerning this population: (i) most of the isolated fungi identified were of *Cladosporium* or *Trametes* affiliation, and yeasts were also present; (ii) bacteria were found to belong to *Actinobacteria*, *Firmicutes*, *Proteobacteria* (*Alpha*, *Beta* and *Gamma* subclasses) and *Bacteroidetes* phyla, with dominant occurrence of the genera *Pseudomonas*, *Sphingomonas*, *Streptomyces*, *Staphylococcus* and *Arthrobacter*. The population of aerobic cultivable bacteria is dominated by strains that are highly similar, regarding their 16S rRNA gene sequence, to some isolates from cold environments, water (sea and freshwater), soil and vegetation. This study demonstrates the great

diversity of microbial population that can be found in clouds, even using only culture-dependent methods.

Comparison of the distribution of Gram-negative vs. Gram-positive bacteria as a function of season shows that summertime, with its higher light exposure, can favour the presence of Gram-negative strains, which are usually more resistant to UV damage. Finally, the numbers of the Gram-negative strains we isolated appear to be generally associated with vegetation that is obviously more present in summertime in Europe. All these parameters lead to a higher proportion of retrieved microorganisms related to Gram-negative strains during the summer.

A very important result of this study concerns the ability of an important proportion of the isolated bacteria to grow at temperatures compatible with those occurring in tropospheric clouds. These results suggest that a great proportion of bacteria present in clouds can be metabolically active in this medium. It is of special interest regarding the potential role of microorganisms in atmospheric chemistry. Work is in progress to test the abilities of these strains to transform organic compounds at low temperatures, and to determine the metabolic pathways involved under these conditions.

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