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Viability and potential for immigration of airborne bacteria from Africa that reach high mountain lakes in Europe

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Summary

We have analysed the diversity of the bacteria, which grow after addition of concentrated airborne particles and desert dust in different microcosms combinations with water samples from oligotrophic alpine lakes. We used, on the one hand, airborne bacteria transported by an African dust plume and collected in a high mountain area in the central Pyrenees (Spain). On the other hand, we collected desert dust in Mauritania (*c.* 3000 km distance, and a few days estimated airborne journey), a known source region for dust storms in West Africa, which originates many of the dust plumes landing on Europe. In all the dust-amended treatments we consistently observed bacterial growth of common phyla usually found in freshwater ecosystems, i.e. *Alpha*-, *Beta*- and *Gammaproteobacteria*, *Actinobacteria*, and a few *Bacteroidetes*, but with different composition based on lake water pretreatment and dust type. Overall, we tentatively split the bacterial community in (i) typical freshwater non-airborne bacteria, (ii) cosmopolitan long-distance airborne bacteria, (iii) non-freshwater low-distance airborne bacteria, (iv) non-freshwater long-distance airborne soil bacteria and (v) freshwater non-soil airborne bacteria. We identified viable long-distance airborne bacteria as immigrants in alpine lakes (e.g. *Sphingomonas*-like) but also viable putative airborne pathogens with the potential to grow in remote alpine areas (*Acinetobacter*-like and *Arthrobacter*-like). Generation of atmospheric aerosols and remote dust deposition is a global process, largely enhanced by perturbations linked to the global change, and high mountain lakes are very convenient worldwide model systems for monitoring global-scale bacterial dispersion and pathogens entries in remote pristine environments.

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Introduction

For a long time ago both African dust outbreaks (Darwin, 1845) and presence of microbes in the air (Pasteur, 1861) have been reported in scientific literature. Today, individual dust outbreaks can be traced on Internet in near-real time from emergence of dust clouds to their reaching site on a global scale and on a daily basis (Herman *et al.*, 1997; Prospero *et al.*, 2002). The Sahara produces more aeolian soil dust than any other desert (Goudie and Middleton, 2001), and satellite images show African dust regularly transported westward over the Atlantic Ocean to North America, South America and the Caribbean, as well as northward across the Mediterranean to Europe (Kellogg and Griffin, 2006). African dust outbreaks can also reach Scandinavia (Franzen *et al.*, 1995), whereas most of the Southern Hemisphere is devoid of major dust activity (Prospero *et al.*, 2002; Pósfai *et al.*, 2003) except for those plumes coming from Australian deserts (Kellogg and Griffin, 2006). Generation of atmospheric aerosols and remote dust deposition is therefore a global process, largely enhanced by perturbations linked to the global change (e.g. Prospero and Lamb, 2003; Moulin and Chiapello, 2006).

The presence of airborne microbes has been largely reported (see a recent review by Kellogg and Griffin, 2006), most of them fungi and bacteria identified by traditional microbiological techniques [i.e. Gram staining, colony-forming units (cfu) on agar plates, and presence of endospores], highlighting the fact that the number of culturable bacteria in air samples consistently increased under African dust events (e.g. Griffin *et al.*, 2001). Several works have also demonstrated that dust clouds may serve not only as a periodic source of nutrients for terrestrial plants and primary producers in nutrient depleted oceanic waters (e.g. Okin *et al.*, 2004; Duarte *et al.*, 2006), but may also serve as a medium for global

transport of microorganisms, including pathogens (Shinn *et al*., 2000; Griffin *et al*., 2001; Brown and Hovmøller, 2002; Prospero *et al*., 2005). Altogether, airborne bacterial fluxes represent an important way for microorganisms to colonize remote environments (Bovallius *et al*., 1978), and satellites providing atmospheric parameters relevant to microbe dissemination into the atmosphere can be used to monitor global transmission of microbes (Simmer and Volz, 1993). Studies dealing with the genetic diversity and community structure, global distributions, potential dispersal and ecological importance of airborne bacteria in natural environments are, however, very limited (Griffin *et al*., 2007; Fierer *et al*., 2008; Hervàs and Casamayor, 2009).

In the present work we have explored the viability and potential for colonization of bacteria transported by an African dust plume reaching the central Pyrenees (Spain). We have analysed in different microcosms experiments the diversity of the bacteria that developed after addition of both concentrated airborne particles and desert dust collected in Mauritania, a known source region for dust storms in West Africa over which large dust storms travel (Goudie and Middleton, 2001). Alpine lakes are generally considered remote and pristine worldwide distributed ecosystems lacking local anthropogenic influence but with recurrent events of remote dust deposition (Psenner, 1999). Recently, it has been shown that the top surface of high mountain lakes (i.e. neuston sampled as reported in Agogué *et al*., 2004) can be a direct interceptor of airborne bacteria from dust outbreaks (Hervàs and Casamayor, 2009), and accumulation of airborne persistent pollutants has also been reported in these pristine environments (Fernández and Grimalt, 2003). These ecosystems respond rapidly to remote environmental perturbations and are very sensitive indicators of global changes (Catalan *et al*., 2006), emerging as very convenient model systems for monitoring global-scale bacterial dispersion.

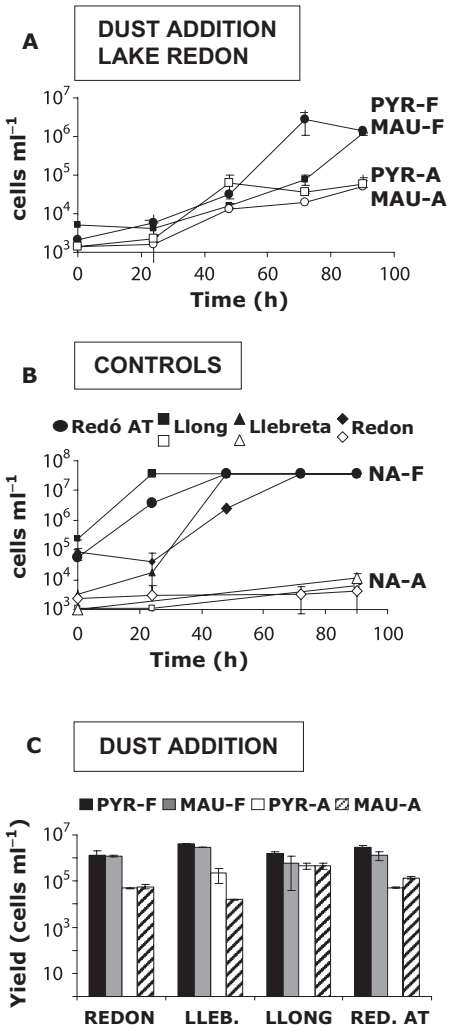

Fig. 1. Bacterial growth (mean value DAPI counts ± standard error from three replicates) in five different microcosms carried out in four alpine lakes. Error bars smaller than dots when no visible. (PYR-) airborne dust; (MAU-) desert dust addition; (-F) filtered lake water; (- A) sterilized lake water; (NA-) artificial nutrients addition. Data for Lake Redon after dust additions in filtered (full markers) and autoclaved lake water (empty markers) (A). Controls carried out for autochthonous bacteria present in filtered (0.2 μm) and autoclaved lake waters after artificial nutrients additions (B). *De novo* formed bacteria (averaged yield from three replicates ± standard error) after airborne and desert dust additions in water samples from four alpine lakes respectively (C).

Results

Bacterial growth in the different microcosms

We carried out a combined set of triplicate microcosms experiments using filtered (< 0.2 μm) and/or sterilized (autoclave) water samples from mountain lakes. The added dust came from atmospheric bulk deposition collected *in situ* in the Pyrenees, and from a Saharan desert soil sample (see *Experimental procedures* section for details). We consistently observed bacterial growth in all the dust-amended treatments using either filtered or autoclaved waters (Fig. 1). Figure 1A shows the example for Lake Redon after additions or airborne particles (PYR) or desert soil (MAU) in triplicate. At the final time, higher bacterial growth was observed in filtered (PYR-F, MAU-F) than in sterilized Lake Redon water (PYR-A, MAU-A), although an exponential response was observed within the first 48 h of incubation in each case. Several controls were carried out to dissect the observed results. First, bacterial growth was not observed either in filtered and autoclaved lake water alone (without dust addition) or after adding simple organic carbon sources (sodium acetate 1 mM, final concentration) (data not shown). Second, we always observed a fast bacterial response

when filtered waters were artificially amended with C, N and P sources [nutrient addition (NA) treatment], even after the first 24 h (Fig. 1B, NA-F treatment). This response was not observed using sterilized lake water as control (Fig. 1B, NA-A treatment). Finally, we also observed that bacteria quickly reacted in filtered lake water after sodium acetate + dust combined addition, reaching high cell concentrations (data not shown). These results showed that dust provided limiting inorganic substrates and that a strong limitation exists *in situ* by non-organic sources, probably phosphorus or/and nitrogen. Overall, we concluded that some local bacteria remained present in the 0.2 μm-filtered water and quickly responded to nutrients inputs, but bacterial contamination was not significantly added during handling in the lab.

For most of the treatments and replicates, we observed that dust additions produced higher number of cells in filtered than in sterilized water samples (up to 1–2 orders of magnitude higher, see Fig. 1C). Total *de novo* formed cells ranged between 3×10^5 and 40×10^5 in PYR-F and MAU-F treatments and between 0.5×10^5 and 4×10^5 in PYR-A and MAU-A. Differences in the produced cells between filtered and autoclaved treatments were significant (Kruskal–Wallis test $P < 0.05$) for Lake Redon and Llebreta, and near significance (Kruskal–Wallis test $P = 0.06$) for Lake Redó AT, but not significant in the case of Lake Llong.

Lake source did not produce selective differences in the final number of bacterial cells produced, and we did not find significant differences among the different lakes between filtered (Kruskal–Wallis test *P*-value 0.3) or autoclaved (Kruskal–Wallis test *P*-value 0.1) enrichments either. Dust origin, in turn, only produced significant difference in the final number of bacteria achieved for autoclaved treatments in Lake Llebreta (i.e. PYR-A versus MAU-A, see Fig. 1C).

Bacterial community assemblages developed in the microcosms

The bacterial 16S rRNA gene sequences obtained from the microcosms belonged to common phyla usually found in freshwater ecosystems, i.e. *Alpha*-, *Beta*- and *Gammaproteobacteria*, *Actinobacteria* and a few *Bacteroidetes*. In most cases, the bands excised and sequenced from the DGGE gels explained more than the 70% of the whole bacterial fingerprinting pattern (Table 1). The remaining percentage corresponded to several faint bands in the DGGE gel that could not be sequenced. The majority of the sequences fitted within different betaproteobacterial clusters (Fig. 2), and we observed separated allocation for the different sequences based on lake water treatments. Thus, within the GSK16 and *Rhodoferax* groups (Beta-I cluster) (Glockner *et al*., 2000; Zwart *et al*., 2002), only sequences from filtered lake water enrichments after either MAU or PYR dust addition were detected. Several clones obtained from a clone library previously carried out in Lake Redon (Hervàs and Casamayor, 2009) were closely related to these samples, but neither sequences obtained from autoclaved treatments nor after artificial nutrients addition on filtered waters were observed within these two groups. Thus, we considered these bacteria as already present autochthonous microbes well adapted to oligotrophic conditions that developed in the treatments using dust supplies, and were called 'Type-1' bacteria, i.e. typically freshwater non-airborne bacteria, not previously reported in airborne or in soil samples (Table 2). Sequences from *Bacteroidetes* were closely related to *Flavobacterium* sp. (97% similarity) and *Flectobacillus* sp. (99%). They were found in filtered water treatments, and had other freshwater bacteria as closest relatives in databases. Therefore, they were also included within 'Type-1'.

Conversely, within the same Beta-I cluster we observed a separated group of sequences closely related among them (98–99% similarity in 16S rRNA gene sequence) obtained from both autoclaved and filtered microcosms for most of the lakes and from desert and airborne dusts additions (labelled 'AIRBORNE-BET' in Fig. 2). Closest relatives in databases (99% similarity) for the AIRBORNE-BET group were detected in worldwide soils, activated sludge and freshwaters, in similar dust enrichment experiments carried out in Sierra Nevada (Granada, Southern Spain) (AM942755, Reche *et al*., 2009), in the clone library of a dusty snow sample in Lake Redon (Hervàs and Casamayor, 2009), and in air samples collected in Colorado (USA) (Fierer *et al*., 2008). Therefore, these

Table 1. Percentage of the DGGE targeted community explained after sequencing bands from the DGGE fingerprints in three lakes for each treatment.

Lake	Treatment	% of the targeted community explained
Redó AT	NA-F	69
	MAU-F	92
	MAU-A	79
	PYR-F	94
	PYR-A	77
Llong	NA-F	71
	MAU-F	80
	MAU-A	54
	PYR-F	84
	PYR-A	74
Llebreta	NA-F	52
	MAU-F	62
	MAU-A	65
	PYR-F	80
	PYR-A	31

(PYR-) airborne dust; (MAU-) desert dust addition; (-F) filtered lake water; (-A) sterilized lake water; (NA-) artificial nutrients addition. More information available in Table S1.

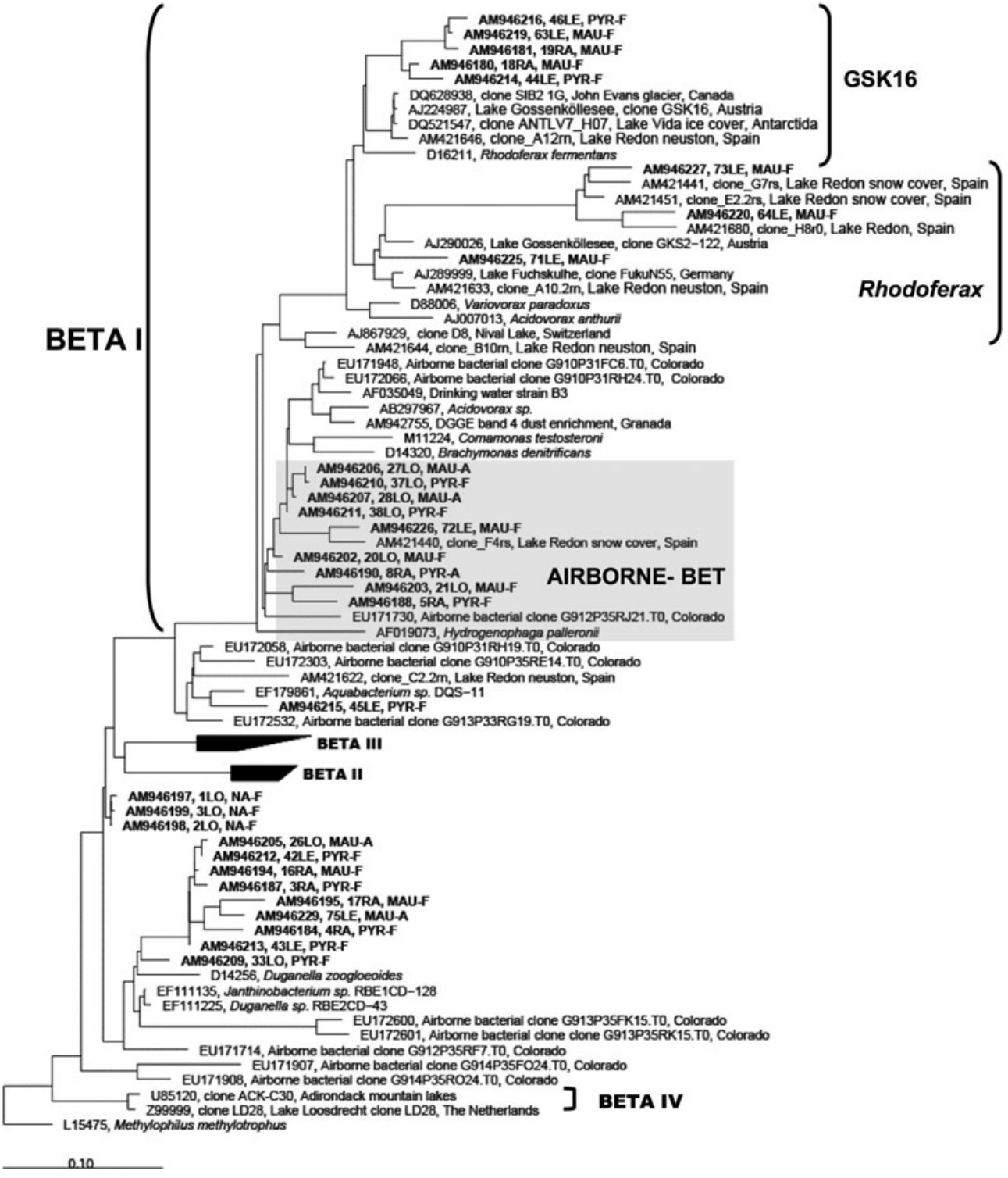

Fig. 2. Maximum likehood phylogenetic tree for 16S rRNA gene of *Betaproteobacteria* with partial sequences inserted by maximum parsimony criterion. Sequences obtained from the three Pyrenean lakes are in bold and accession numbers in GenBank are indicated. (RA) Lake Redó AT, (LO) Lake Llong, (LE) Lake Llebreta. (PYR-) airborne dust; (MAU-) desert dust addition; (-F) filtered lake water; (-A) sterilized lake water; (NA-) artificial nutrients addition. See text and Table 2 for the meaning of shading clusters (98% similarity). Scale bar: 0.10 mutations per nucleotide position.

Table 2. Presence/absence of different taxa found in five different microcosms carried out in different alpine lakes.

Type	PYR-F	MAU-F	PYR-A	MAU-A	NA-F	Freshwater	Airborne	Soil	Taxa
1	+	+	ND	ND	ND	+	ND	ND	GKS-16, *Rhodoferax Bacteroidetes*
2	+	+	+	+	ND	+	+	+	Airborne-BET
3	ND	ND	ND	ND	+	ND/+	+	+	NA-F Beta/ NA-F Pseudomonas
4	ND	ND	+	+	ND	ND	+	+	Airborne-ACN Airborne-ACT
5	+	ND	+	ND	ND	+	+	ND	Airborne-ALF

(PYR-) airborne dust; (MAU-) desert dust addition; (-F) filtered lake water; (-A) autoclaved lake water; (NA-) artificial nutrients addition. Freshwater, airborne and soil 16S rRNA gene sequences detected after a BLAST search in GenBank are also indicated.
Typologies: Type 1: freshwater non-airborne bacteria. Type 2: cosmopolitan long-range airborne bacteria. Type 3: non-freshwater low-range airborne cosmopolitan bacteria. Type 4: non-freshwater long-range airborne soil bacteria. Type 5: freshwater non-soil airborne bacteria. ND, not detected. See text for detailed characteristics of each bacterial type.

were cosmopolitan long-distance airborne bacteria, i.e. bacteria that are viable after long distances transport in the air, present in a wide range of freshwaters and soils ('Type-2' bacteria in Table 2). Curiously, these bacteria have not been detected so far in the molecular surveys carried out in alpine lakes.

Another group of *Betaproteobacteria* sequences present in both filtered and autoclaved treatments and in several of the lakes were closely related (98% similarity in the 16S rRNA gene) to *Duganella zoogloeoides* (formerly *Zoogloea ramigera*) (Fig. 2, bottom), and 99% similar to freshwater bacteria found in the mesotrophic Lake Schoehsee, Germany (AJ556800), in groundwater in Japan (AB237673), and in the Chesapeake Bay, USA (EU802204). No closest relatives from oligotrophic lakes appeared in the database. These bacteria were not detected in the PYR-A treatments (i.e. airborne dust and sterilized freshwater) but appeared in the combination MAU-A. Altogether, this may indicate we were dealing with freshwater bacteria of soil origin and either they probably were not resistant to long air journeys or we failed to detect them in the airborne dust microcosms (see *Discussion*). This *Duganella*-like group has not been detected in the molecular surveys carried out so far in alpine lakes, and could be considered closer to 'Type-2' than to 'Type-1', bacteria.

A small group of *Betaproteobacteria* sequences obtained from the NA-F treatments (artificial nutrients mixture added) were grouped outside the Beta-I, -II and –III clusters and had as closest relatives (99% similarity) sequences obtained from human fluids and soils (see accession numbers DQ188572, AF499897, AF529336 in GenBank). These are viable bacteria already present in the alpine lakes but that probably never found appropriate conditions to develop. We called them 'Type-3' bacteria, probably of allochthonous origin and high nutrient demand, and were considered as non-freshwater bacteria.

Gammaproteobacteria sequences were recovered from microcosms with sterilized lake water after desert and airborne dust additions (MAU-A and PYR-A within the *Acinetobacter*-like cluster, labelled AIRBORNE-ACN in Fig. 3), and from NA treatment on filtered lake water (*Pseudomonas*-like, Fig. 3). Curiously, *Gammaproteobacteria* were not detected in any filtered lake water after dust additions. The *Acinetobacter*-like cluster (99% similarity) also contained sequences obtained from a related study carried out in 2005 in a high-altitude reservoir in southern Spain (Sierra Nevada) using airborne dust collected *in situ* (AM942756, Reche *et al*., 2009), and from a clone library carried out in 2004 in a dusty snow samples collected in Lake Redon (Hervàs and Casamayor, 2009). Thus, viable *Acinetobacter* spp. were detected all along the gradient from dust emission in Mauritania to the Pyrenees (including the middle station of Sierra Nevada) and along different years, but were not a typical freshwater bacterioplankton component. We called them 'Type 4' bacteria, i.e. long-distance airborne soil bacteria that easily resist journeys in the troposphere but do not successfully colonize freshwater environments. Sequences related to *Pseudomonadaceae* were within a diverse cluster of *Pseudomonas*-like sequences (97–99% similarity) previously detected in soil, ice, freshwaters and airborne samples. As in the case of the NA-F *Betaproteobacteria*, here the *Pseudomonas*-like bacteria probably are of allochthonous origin and high nutrient requirements that remain viable in oligotrophic freshwater environments as a minor component (i.e. 'Type 3' bacteria).

Alphaproteobacteria were detected in both filtered and sterilized lake water microcosms mixed with airborne dust samples (PYR-A, PYR-F, respectively, AM946191), but not in the microcosm with soil desert added. We labelled this group as AIRBORNE-ALF (Fig. 3), and they were related (96% similarity) to *Sphingomonas* genus and to clones obtained from air samples (EU171836, Fierer

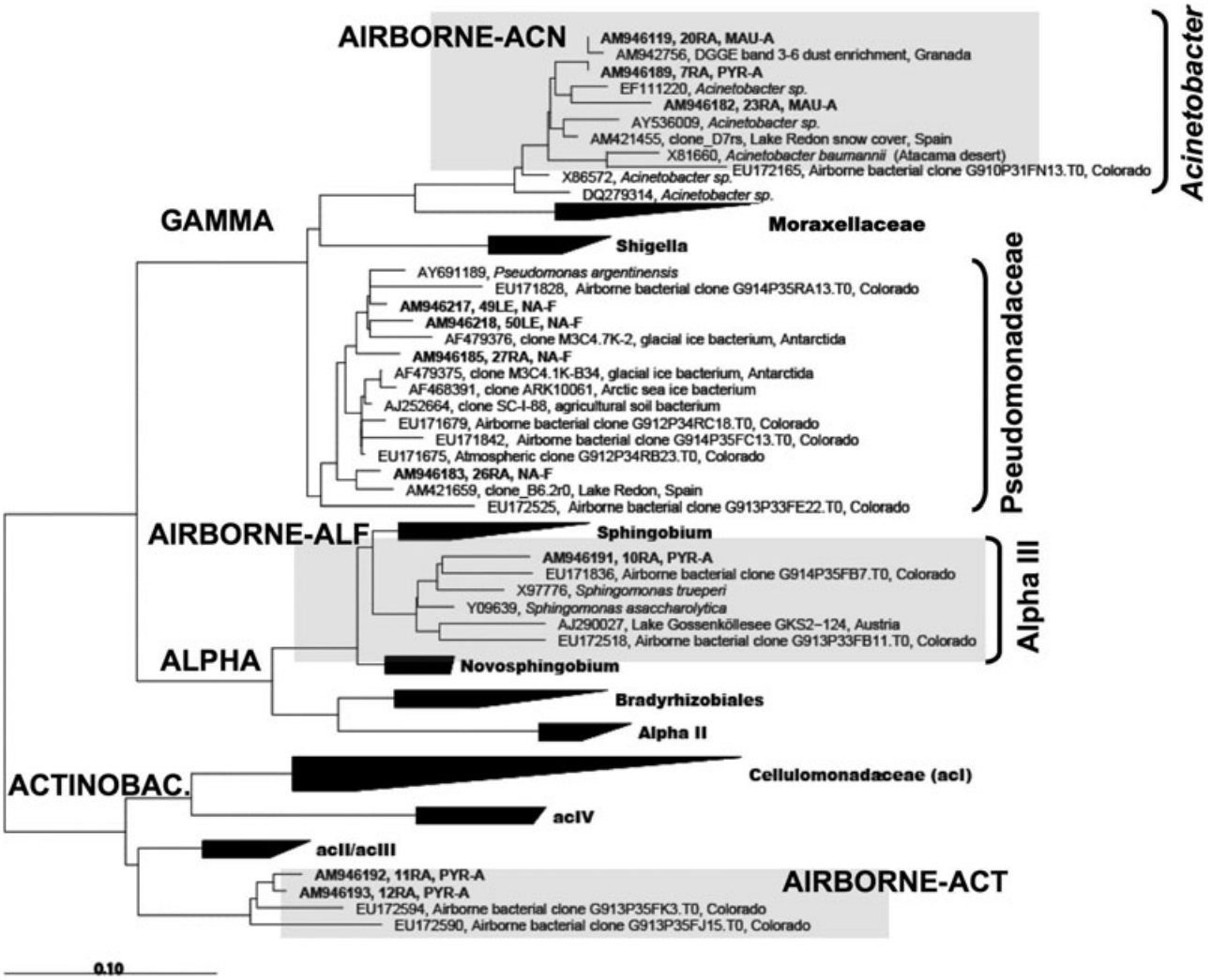

Fig. 3. Maximum likehood phylogenetic tree for 16S rRNA gene of *Gamma-* and *Alphaproteobacteria*, and *Actinobacteria* with partial sequences inserted by maximum parsimony criterion. Sequences obtained from the three Pyrenean lakes are in bold and accession numbers in GenBank are indicated. (RA) Lake Redó AT, (LO) Lake Llong, (LE) Lake Llebreta. (PYR-) airborne dust; (MAU-) desert dust addition; (-F) filtered lake water; (-A) sterilized lake water; (NA-) artificial nutrients addition. See text and Table 2 for the meaning of shading clusters (98% similarity). Scale bar: 0.10 mutations per nucleotide position.

et al., 2008), within the previously described freshwater Alpha-III cluster (Glockner *et al*., 2000). *Alphaproteobacteria* are more often found in seawater (Glockner *et al*., 1999; A. Barberan and E.O. Casamayor, unpublished data), but previously we had detected *Alphaproteobacteria* within the *Sphingobium* cluster in Lake Redon (AM950231 and AM950232). *Sphingomonas* spp. have also been cultured from Caribbean air samples carrying African desert dust (Griffin *et al*., 2001), and it is a genus that contains potential plant and animal (coral) pathogens. Thus, we classified AIRBORNE-ALF as 'Type 5' bacteria, i.e. long-distance airborne bacteria usually not found in soils, but that sporadically could colonize oligotrophic freshwater environments.

Finally, *Actinobacteria* were also obtained from airborne dust samples on both filtered and autoclaved lake water microcosms and were related (97% similarity) to *Arthrobacter*-like sequences and other soil bacteria, and to clones obtained from air samples (Fig. 3). Interestingly, they were not related to the most common *Actinobacteria* found in freshwaters (i.e. cluster ac-I) also present in Lake Redon (Hervàs and Casamayor, 2009). *Arthrobacter* sp. is a cosmopolitan group also found in saline environments, such as salt soils and salterns and seawater neuston (Casamayor *et al*., 2000; Agogué *et al*., 2005). We labelled *Arthrobacter*-like sequences as AIRBORNE-ACT, and we also classified them as 'Type 4' bacteria, i.e. long-distance airborne bacteria usually found in soils but that do not colonize oligotrophic freshwater environments.

Overall, in our study we identified four clusters where viable airborne bacteria were present, ranging from those with potential for freshwater colonization (AIRBORNE-

BET and AIRBORNE-ALF) to those where freshwater representatives have not previously been reported (AIRBORNE-ACN and AIRBORNE-ACT). In addition, we identified non-airborne bacterial phylotypes ranging from strictly freshwater bacteria (e.g. Type 1) to those detected in oligotrophic freshwaters, but that probably never will find appropriate conditions to actively grow *in situ* (Type 3). Within the airborne bacteria, some showed no abilities to grow in oligotrophic freshwaters (e.g. Type 4), whereas some others had the potential to colonize remote alpine lakes (Types 2 and 5). Of course, we cannot rule out other typologies, and/or the presence of other bacterial phylotypes within the typologies considered as many more bacteria could have developed below the detection limit of the molecular approach used in our study (0.1–1% of total targeted cells, Casamayor *et al*., 2002).

Discussion

The dust annually mobilized to the atmosphere has been estimated in more than one billion metric tons (Moulin *et al*., 1997) that actively exchanges biological material among ecosystems across continents. Direct counts of bacteria-like and virus-like particles present in the air have been shown to be 10 times higher in the presence of dust events than during normal conditions (*c.* 20 cells per litre of air, Griffin *et al*., 2001). Altogether, up to 10^{18} bacterial cells per year are globally transported by aerosols (Griffin *et al*., 2002). Although this number represents only a small portion of the total bacterial numbers estimations (Whitman *et al*., 1998), atmospheric transport probably plays a key role for global bacterial dispersion. Most of the time, dust plumes are approximately 5 km high in the troposphere and submitted to strong desiccation and UV doses. Dust particles may provide, however, shadow protection and capture atmospheric humidity helping bacteria to keep alive (Tong and Lighthart, 1998; Gorbushina *et al*., 2007). In fact, many of the airborne bacteria we have recovered from dust collected in the Pyrenees belonged to bacterial groups where presence of resistance forms against nutrient depletion, desiccation, radiation, and temperature fluctuations, such as endospores, have not been reported (e.g. *Alpha-* and *Betaproteobacteria*). In turn, typically endospores-forming *Firmicutes* (e.g. *Bacillus* sp.) were not detected in our enrichments although in the sporulated forms of different *Bacillus* species isolated from the air, spores germination happens only after 30 min incubations in water (Maron *et al*., 2005). Thus, for this group of Gram-positive bacteria cell multiplication seems to be not favoured in high mountain oligotrophic waters. This is in agreement with the fact that *Firmicutes* are usually not found in 16S rRNA gene surveys from surface freshwater bacterioplankton (they were present in less than 3% of total sequences from a global survey carried out in databases, A. Barberan and E.O. Casamayor, unpublished data) neither in a previous work in Lake Redon (Hervàs and Casamayor, 2009). Other resistance strategies are therefore expected for the non-sporulated airborne bacteria for maintaining viability along the journey.

Viability of long-range airborne bacteria has traditionally been determined after plate counts (cfu) on nutrient agar media (see a recent review by Kellogg and Griffin, 2006). The spectrum of cultured isolates is narrower than the diverse bacterial lineages detected using DNA-based molecular methods. For instance, using culture-independent 16S rRNA gene cloning and sequencing of directly collected air samples, the groups mostly found were Gram-positive bacteria (i.e. *Actinobacteria*, *Bacillus* and other *Firmicutes*), but *Alpha-*, *Beta-* and *Gammaproteobacteria* have also been reported (Radosevich *et al*., 2002; Wilson *et al*., 2002; Maron *et al*., 2005; Fierer *et al*., 2008). In turn, most of the genera cultured from the air in different worldwide studies are Gram-positive organisms (up to 90%, Shaffer and Lighthart, 1997) with *Bacillus* spp. as the genus more abundant and represented at all locations (e.g. Di Giorgio *et al*., 1996; Prospero *et al*., 2005; Gorbushina *et al*., 2007). It is a well-known fact that cultured bacteria appear to constitute only a small fraction of the total cell counts in airborne samples (less than 1%, e.g. Griffin *et al*., 2001). However, studies at the DNA level can detect both bacteria alive that cannot grow on agar plates but also dead microbes attached to dust particles. Of course, bacteria can also be present in cryptobiotic resistance forms (exhibit no signs of life) for which direct DNA extraction is difficult to achieve, and would remain missing or underrepresented just after favourable conditions make spores germinate and produce vegetative cells (Maron *et al*., 2005).

The experimental design carried out in the microcosms studied here facilitated cell activation and growth in conditions closer to the oligotrophy prevailing in alpine lakes, and the viable cells enriched were more similar to bacteria detected by direct DNA extraction than to those recovered in nutrient-rich agar media. Nevertheless, ecological implications should be carefully extrapolated from these experiments because potential local competitors and predators were excluded from the microcosms, and incubation conditions did not mimic local conditions (UV and light regimes, daily marked changes in temperature, among others). The results of the conducted experiments indicated a potential for immigration of specific taxa, but whether or not Sahara dust settling on the surface of the investigated lakes is really causing any successful invasion of African bacteria remains to be examined following the fate of these bacteria *in situ* at the right temporal scale, and with the combined use of other molecular tools such as fluorescence *in situ* hybridization.

Dust-associated bacteria probably were directly derived from soils when the dust was mobilized by winds. But bacteria could have also been injected into dusty air masses from local sources (including seawater or vegetation) by advection and vertical mixing in convective clouds or being independently suspended in the air and washed out by the dust outbreak. This may be the case for the AIRBORNE-ALF found in the alpine lakes, an airborne group not detected in soils but with close relatives in the ocean and on plants. Interestingly, we found high similarity between bacteria enriched from the original source in Mauritania (MAU-A treatments) and those obtained by Kellogg and colleagues (2004) in agar plates from the neighbouring country Mali. For instance *Arthrobacter* sp., *Acinetobacter* sp. and *Duganella zoogloeoides*-like bacteria were found both by Kellogg and colleagues in R2A agar, and in our own microcosms. In addition, we found closely related or even the same phylotypes in Mauritian desert soil and in the airborne dust collected in the Pyrenees (e.g. AIRBORNE-BET, AIRBORNE-ACN, AIRBORNE-ACT). These results support the African origin of the airborne dust present in high mountain lake areas of the Pyrenees but also indicated the potential for immigration in pristine aquatic ecosystems that certain foreign bacterial groups may have. We will focus on two examples with more detail in the next paragraph, noting that the microbes identified are a subset of the total viable organisms detected, not a complete catalogue.

First, microorganisms detected both in the origin and in the sink but not in airborne samples, such as *Duganella*-like, could probably be related to historical factors that continuously enrich the seed bank of viable but rare (very low abundant) bacteria in alpine lakes. In fact, it is well known that African microorganisms have been exported to Europe and other continents with desert dust for centuries. Alternatively, we cannot rule out that dark incubations carried out in this work with airborne samples would have inhibited photoreparation mechanisms by light-dependent photolyases (Carell *et al*., 2001) required after the atmospheric journey to facilitate viability of some of the cryptobiotic cells. In the second example, one bacterium apparently not well equipped for harsh long-distance air transport, such as *Acinetobacter* sp. (or even *Sphingomonas* sp.), surprisingly shows successful dispersion rates and cosmopolitan distribution. We found very closely related *Acinetobacter* spp. at three different locations (Mauritania, Sierra Nevada and Pyrenees) and at three different years (Hervàs and Casamayor, 2009; Reche *et al*., 2009), indicating that this is a frequent and viable airborne bacterium with the potential to develop in lake water. This bacterium, however, would require changes on the prevalent *in situ* conditions to successfully develop and out-compete locally adapted bacteria. Successful colonization in these environments for a bacterium is a complex issue to solve involving limiting factors (e.g. temperature or ionic composition), competition with local bacteria and resistance to predators and to virus attack, among others (Pernthaler, 2005 and references therein; Reche *et al*., 2009). We have not explicitly deal with this in the present paper, but the whole set of viable airborne cells with the potential to develop in oligotrophic lakes is certainly large, and we cannot rule out future success giving the fact that these fragile ecosystems are biologically reactive to global changes in temperature or eutrophication (Psenner, 1999). The case of *Acinetobacter* and *Sphingomonas* can also illustrate potential survival and dispersion strategies for pathogens. The *Acinetobacter*-like genus contains opportunistic highly resistant human pathogens (Bergogne-Berenzin and Towner, 1996) and although we did not specifically look for pathogens in this work, it is reasonable to assume that some wind transported pathogens species could have remained viable over great distances, protected by the same mechanisms that protected these bacteria. In fact, opportunistic human, plant and animal pathogens are very common in airborne dust samples (Griffin *et al*., 2001; Kellogg *et al*., 2004).

Nutrients supply (nitrogen and phosphorus, among others) by airborne dust on terrestrial and oceanic ecosystems has been extensively explored (e.g. Rodà *et al*., 1993; Psenner, 1999; Herut *et al*., 2002; Bonnet *et al*., 2005; Morales-Baquero *et al*., 2006), but the present knowledge of the effects on the bacterioplankton is quite limited (Reche *et al*., 2009). On the one hand, in the alpine lakes highly influenced by Saharan dust outbreaks, atmospheric deposition consistently stimulated bacterial growth probably by phosphorus inputs (Pulido-Villena *et al*., 2008), and we have found a similar fertilization response in all four Pyrenean lakes after dust supply either from the source or from the sink. On the other hand, airborne dust also contains organic carbon (Eglinton *et al*., 2002; Usher *et al*., 2003) that could be used as carbon source for heterotrophic bacteria although probably this carbon supply is of limited relevance in our lakes. The initial tests indicated that planktonic bacteria were not carbon-limited (acetate additions did not show bacterial response) in the four near lakes. The lower number of cells obtained in the microcosms with sterilized water is in the same line of reasoning. Probably, the high pressures and high temperatures in the autoclave could have also modified the quality and quantity of the organic matter (Wilson *et al*., 2000), sizing down the final bacterial yields. In addition, no significant differences in bacterial growth were found between the desert dust and the airborne dust after a journey into the troposphere that would have modified organic components (Usher *et al*., 2003). Altogether, it seems that dust basically would supply bacteria with inorganic limiting nutrients (mainly phosphorous and nitro-

gen, Pulido-Villena *et al*., 2008) to use the available dissolved organic carbon already present in these lakes.

Overall, alpine lakes and the bacterioplankton inhabiting there are a good scenario for combined research of physical and meteorological data (long distance dust transport, level of protection on dust particles and nutrients supply) with biological responses (ecological limitations for bacterial dispersion, survival and colonization). High mountain lakes are among the ecosystems with larger similarities throughout the planet and with a high level of bacterial 'cosmopolitanism' (Sommaruga and Casamayor, 2009) offering research opportunities beyond what could be expected from their quantitative relevance in the Earth system (Catalan *et al*., 2006). As changes in climate will presumably lead to an alteration in dust export (e.g. in frequency and intensity) and will induce ecosystem-level changes, high mountain lakes appear as particularly suitable model systems to explore dispersal mechanisms, survival/colonization abilities, competition and local adaptations of airborne microorganisms at global scales in a changing world.

Experimental procedures

Water and dust samples collection

We carried out a combined set of microcosms experiments using water samples from four selected high mountain lakes in the Pyrenees. Dust obtained from two different sources, i.e. a Mauritanian sandy soil located within the Sahel region 40 km south-east of Boûmdeid in the Karakoro river basin (17.21°N, 11.12°E; West Africa), and atmospheric bulk deposition collected along a week in May 2007 after a Saharan dust outbreak reaching the Pyrenees area (42.55°N, 0.88°E; North Spain) (http://www.calima.ws) were used.

The lakes were in the Spanish Pyrenees within a few kilometers distance and located along an altitudinal gradient with different sizes and catchment areas (Auguet and Casamayor, 2008). Lake Redon (42°38′33″N, 0°46′13″E) is 2240 m.a.s.l. (meters above sea level), 24 ha size, catchment area 153 ha, and has been extensively studied for the last 25 years (Catalan *et al*., 2006). The remaining three lakes are within the same watershed in a protected area in the Aigüestortes National Park, i.e. Lake Redó d'Aigüestortes (2117 m.a.s.l., 6 ha size, 325 ha catchment area), Lake Llong (2000 m.a.s.l., 7 ha size, 1111 ha catchment area) and Lake Llebreta (1620 m.a.s.l., 8 ha size, 5438 ha area). Water samples were collected from the surface of the lakes and kept in 5 l bottles in the dark. After *c*. 2 h, the complete volume was filtered twice by 0.2 μm polycarbonate membranes to exclude most of the local microbes, and kept at 4°C. A first set of filtered water was directly used for enrichment experiments mixed with dust samples. A second set was previously sterilized by autoclaving for 30 min at 120°C, and used in parallel.

The Mauritanian sandy soil was initially treated with an automatic sifter Biometa AS 200 (Biometa Tecnología y Sistemas S.A., Asturias, Spain) for 15 min with 30 s intervals and 0.75 mm g^{-1} amplitude. The different sifters were previously sterilized under UV for 30 min. The < 0.63 μm dust fraction was collected and further used for bacterial enrichments. The Pyrenean aerosol was obtained from an automatic dry/wet passive collector MTX ARS 1010, 667 cm^2 area, polyethylene vessel (MTX, Bologna, Italy) placed on the vicinity of Lake Llebreta along a Saharan intrusion dust event followed by TOMS (Total Ozone Mapping Spectrometer, http://toms.gsfc.nasa.gov/aerosols/aerosols_v8.html). TOMS provides a measure of the atmospheric loading of UV-absorbing aerosols (i.e. mineral dust and soot from anthropogenic and natural combustion sources) (Herman *et al*., 1997) over both water and land surfaces and thereby yields information on terrestrial dust sources (Prospero *et al*., 2002). Dust particles were collected onto precombusted (450°C, 4 h) Whatman GF/F filters. Next, filters were dried in a laboratory heater for 4 h and kept in a dark and dry place.

Enrichment experiments

Filtered (F) and autoclaved (A) water samples were submitted to three independent treatments (NA, MAU and PYR) in triplicate microcosms (3 × 250 ml each). In the NA treatment, lake water was directly supplemented with organic carbon (sodium acetate 1 mM, final concentration), nitrogen (casamino acids 0.04% w/v, final concentration) and phosphorous (KH_2PO_4 10 μM, final concentration) respectively. Nutrient addition served both to check for autochthonous bacteria present still in the < 0.2 μm filtered sample (i.e. NA-F samples), and to detect contaminant bacteria introduced after handling in the laboratory (NA-A samples). For the 'MAU treatment', up to 8 mg l^{-1} (final concentration) of Mauritanian desert dust fraction < 0.63 μm was added. This is approximately a daily dose collected in the same type of passive collector in south Spain during an extreme African dust intrusion event and within the range reported in the literature for similar experiments (Reche *et al*., 2009 and references therein). Finally, for the 'PYR treatment' we used 1/8 of the total wet deposition collected in the Pyrenean collector along 1 week. Each treatment was sonicated for 5 min in a Branson 3510 ultrasonic water bath (Branson Ultrasonics, Danbury, Connecticut, USA) to split dust aggregates, and microcosms were incubated in a thermostatic chamber at 16°C in the dark for 90 h. Small water volumes (15 ml) were taken at five times (initial, every 24 h and final time) fixed in formaldehyde at 4°C for 1 h, and filtered on 0.2 μm polycarbonate filters for DAPI (4,6-diamidino-2-phenylindole) staining and microscopic counts in triplicates. At the final time, the remaining volume (*c*. 200 ml) was filtered on 0.2 μm pore polycarbonate filters for DNA extraction. Controls carried out in several occasions on filtered and autoclaved lake waters without additions did not show detectable bacterial growth.

Samples processing

Counting of DAPI-stained cells was done with an epifluorescence Axioplan microscope (Zeiss, Jena, Germany) with enough cells and fields for statistical treatment. Statistical analyses were carried out with Stadtgraphics version XV centurion. DNA extraction was carried out in lysis buffer (40 mM EDTA, 50 mM Tris pH 8.3, 0.75 M sucrose) as

previously described (Dumestre *et al*., 2002) after lysozyme, proteinase K and sodium dodecyl sulfate incubation, followed by phenol extraction and ethanol precipitation. Bacterial 550 bp 16S rRNA gene fragments suitable for DGGE analysis were PCR-amplified with the primer combination 358F with a GC clamp (40-nucleotide GC-rich sequence, 5′-CCT ACG GGA GGC AGC AG-3′) and 907RM (5′-CCG TCA ATT CMT TTG AGT TT-3′) (Casamayor *et al*., 2002) and DGGE fingerprints were run as described (Casamayor *et al*., 2002) for 5 h at a constant voltage of 200 V and at 60°C in a 30–70% vertical denaturant gradient (the 100% denaturant agent is 7 M urea and 40% deionized formamide). Gels were photographed with UV transilumination after Sybr GOLD staining, and most of the bands were excised, re-amplified and purified for sequencing as reported (Estrada *et al*., 2004). The 16S rRNA gene sequences were submitted to a BLAST Search (http://www.ncbi.nih.gov/BLAST) for preliminary identification, and were further aligned with the ARB program package (Ludwig *et al*., 2004). Partial sequences (*c*. 500 nt) were inserted into a validated tree provided by default (Greengenes database, reference tree calculated by maximum likelihood with sequences > 1250 nt length, DeSantis *et al*., 2006) by the maximum parsimony criterion without allowing changes in the overall tree topology. Sequences were deposited in GenBank under accession numbers AM946178 to AM946229.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Closest relatives for the 16S rRNA gene sequences obtained from the excised DGGE bands in the different microcosms. (LE) Lake Llebreta, (LO) Lake Llong, (RA) Lake Redó AT. (PYR-) airborne dust addition; (MAU-) desert dust addition; (-F) filtered lake water; (-A) autoclaved lake water; (NA-) artificial nutrients addition. (None) No cultured strain closer than 95% found in the database.

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