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Detection and phylogenetic analysis of coastal bioaerosols using culture dependent and independent techniques

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Abstract

Bioaerosols are emerging as important yet poorly understood players in atmospheric processes. Microorganisms in the atmosphere have great potential to impact chemical and physical processes that influence global climateby participating in both ice nucleation and cloud droplet formation. The role of microorganisms in atmospheric processes is thought to be species-specific and, potentially, dependent on the viability of the cell; however, few simultaneous measurements of both parameters exist. Using a coastal pier monitoring site as a sampling platform to investigate the exchange of airborne microorganisms at the air-sea interface, culture independent (i.e. DNA clone libraries from filters) and culture dependent approaches (i.e. agar plates) were combined with 18S rRNA and 16S rRNA gene targeting to determine the microbial diversity. The results indicate that in these coastal air samples two fungal phyla, Basidiomycota and Ascomycota, predominate among eukaryotes while Firmicutes and Proteobacteria predominate among bacteria. Furthermore, our culture dependent study verifies the viability of microbes from all four phyla detected through our culture independent study. Contrary to our expectations and despite oceanic air mass sources, common marine planktonic bacteria and phytoplankton were not abundantly found in our air samples indicating the potential importance of bioaerosols derived from beaches and/or coastal erosion processes.

1 Introduction

Bioaerosols are abundant in the atmosphere originating from both marine and terrestrial environments. Bioaerosols include plant debris, pollen, fungi, bacteria, and viruses. Of all these, fungi and bacteria are the best characterized. The global contribution of bioaerosols to atmospheric particles is estimated to be 1000 Tg/yr (Deguillaume, 2008). Over land, dry and windy conditions contribute to aerosol formation with an estimated 25–30% of continental aerosol estimated to originate from biological

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sources (Morris, 2004; Matthia-Maser, 2000). In the marine atmosphere, about 10% of particulate matter by volume is attributed to biological material (Matthias-Maser, 1998) with the primary mechanism of introduction to the atmosphere occurring via bubble bursting at the sea surface (Aller, 2005). Measurements of fungal spore emissions, estimated to be 50 Tg/yr, indicate that Ascomycota and Basidiomycota are the major contributors to these primary biological aerosols (PBA) (Elbert, 2007). Over terrestrial environments, bacterial PBA have been estimated to be greater than 1×10^4 /m^3 (Bauer, 2002) with 1500 /ml being detected in cloud droplets (Sattler, 2001). Overall, these studies demonstrate the abundance and diversity of biological material in the atmosphere, suggesting a potential role for bioaerosols in global climate and the hydrological cycle.

Bioaerosols are thought to influence atmospheric processes by participating in atmospheric chemistry and cloud formation. Bacteria and fungal spores are known to contribute to the organic carbon content of cloud water and aerosols (Bauer, 2002) through the contribution of polysaccharides such mannitol, glucose, fructose (Elbert, 2007). The contribution of organic species to aerosol composition is significant because organic species with differing solubilities can exert either a strong positive and negative effect on the hygroscopic properties of aerosols (Saxena et al., 1995). In addition to impacting aerosol chemical composition, bioaerosols act as ice (IN) and, to a lesser extent, cloud condensation nuclei (CCN) (Möhler, 2007). Furthermore, Pratt et al. (2009) recently detected biological particles in situ in cloud ice residues demonstrating the importance of bioaerosols to cloud formation.

The CCN activity of PBA has been studied with pollen, fungi, and more extensively with bacteria. Bauer et al. (2003) found that a wide range of bacterial species were active as CCN at supersaturations between 0.7–0.11%. As IN, some bioaerosols are active at relatively high temperatures between −2 °C and −9 °C (Georgakopoulos, 2009) initiating the early onset of freezing. Not all PBA, however, have been shown to nucleate ice crystals. The ability of organisms to act as IN has been suggested to be highly species dependent with the nucleating activity of bacteria arising from the presence of

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a surface protein that acts as a template for ice crystallization (Morris, 2004). Protein variants from several bacteria, including the well known ice nucleating *Pseudomonas syringae*, have been studied and grouped according to the temperature at which ice nucleation is more efficient (Möhler, 2007). Since different organisms have distinct ice crystal and cloud condensation capabilities, it is important to characterize PBA in specific atmospheric environments to understand how and to what extent bioaerosols due to specific bacteria impact global climate.

The role of PBA in atmospheric processes is complicated by the potentially different impacts that non-viable and metabolically active microorganisms have on atmospheric processes. Observations have shown that metabolically active cells in the air can directly participate in atmospheric chemistry. Viable microorganisms have been detected in Antarctic cloud water (Saxena, 1983), and in cloud droplets collected at high altitude, where bacteria were found to be actively growing at temperatures of 0 °C and below (Sattler, 2001). Evidence presented by Amato et al. (2007) is also suggestive that large portions of bacteria may be metabolically active in clouds. Many of these microbes have been found to have important metabolic properties for degrading major cloud water organic compounds such as formate, acetate, lactate, methanol, and formaldehyde (Amato, 2005; Deguillaume, 2008). Furthermore, the efficiency of microorganisms to serve as IN has been suggested to be highly dependent on the status of the cell. Proteins associated with intact cells have been suggested to be more efficient at ice nucleation than purified proteins or proteins associated with disrupted cells (Möhler, 2007; Morris, 2004). Thus not only is the detection and identification of bioaerosol populations required in various environments, but information regarding the viability and metabolic activity of these organisms is also desirable for ascertaining the role of different microorganisms on atmospheric chemistry, cloud droplet formation, and ice crystal formation.

Although the impact bioaerosols have on climate is becoming evident, comprehensive investigations of the microbial populations present in the atmosphere and how this might be influence by changing environmental conditions is still necessary. Previous

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work has generally relied on culture based techniques that provide insight into the viable organisms that comprise bioaerosols; however, limitations arise due to biases created by the narrow range of growth supported by specific media. One method of detecting organisms, without growth media biases, involves the use of molecular biology techniques on bioaerosols collected via impaction onto filters followed by direct gene targeting on environmental samples. Airborne bacteria and fungi have been successfully characterized using 16S rRNA and 18S rRNA gene analysis, respectively (Nehme, 2008; Zhihong Wu, 2003). A limitation to culture independent techniques is that it does not provide information regarding cell viability. In this study, we incorporate gene targeting using both culture dependent and independent techniques to analyze the distribution of microorganisms found at an ocean-land-atmospheric interface. Sampling was performed at a coastal monitoring pier to investigate if common marine microorganisms were aerosolized from seawater or if coastal bioaerosols originated mainly from terrestrial sources.

2 Materials and methods

2.1 Culture independent air sampling and DNA isolation

All air samples were collected on a pump house rooftop located on the end of the Scripps Institution of Oceanography (SIO) pier. The end of the pier is approximately 1000 feet from shore. What is the height of the pier from the ocean? Three sets of bioaerosols were collected using a 10-stage micro-orifice, uniform-deposit impactor (MOUDI) Model 110 (Applied Physics Inc.) with size cut-point diameters at 18, 10, 5.6, 3.2, 1.8, 1.0, 0.56, 0.32, 0.18, 0.10, and 0.056 µm using Fluoropore membrane PTFE filters (Millipore). Sampling equipment and stages were cleaned with methanol before each sampling event. Air was drawn into the MOUDI through a stainless steel sampling mast that is 8′ in height and 1/2″ in diameter. The sampling conditions of the three filters sets (filter sets A–C) can be found in Table 1. Immediately after collection,

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the filters were stored at −80 °C until subsequent analysis. For Set A, DNA isolation was performed on different size cut filters, but only the 5.6–10 µm cut points yielded significant DNA amounts compared to control filters and showed PCR amplification. From Set B, filters with visible particulate material were selected for DNA isolation (filter size cut range 0.32–3.2 µm), DNA was combined, and PCR was done on combined DNA. From the remaining size cuts, DNA was also isolated but did not yield significant DNA and did not produce PCR amplification. For Set C, particulate material was visible in filters with size cuts 0.18–0.56 µm; the DNA from these filters was also combined and amplified through PCR. Other filters outside the 0.18–0.56 µm range did not yield significant DNA and did not show PCR amplification. To isolate all DNA, filters were re-suspended in TE buffer (50 mM Tris, 20 mM EDTA), homogenized through bead-beating, and subjected to phenol chloroform extractions and ethanol precipitation (Palenik, 2009). Purified DNA was quantified using Quant-it PicoGreen dsDNA Kit (Invitrogen).

2.2 Air mass back trajectory analysis

Air mass back trajectories for each sample set were obtained using the NOAA/HYSPLIT model 4.8 (Draxler, 2003). Set A and B samples were collected from oceanic air masses, Set C from a continental air mass, and Set D from a mixed continental-oceanic air mass. The atmospheric conditions for each sampling event are found in Table 1 and back trajectory images in Fig. 1.

2.3 18S rRNA amplification, cloning, and sequencing

Filter Set A was subjected to PCR amplification and cloning to construct an environmental library of 18S rRNA gene regions. For PCR, the primers used were 5′-ACCTGGTTGATCCTGCCAG-3′ and 5′-TGATCCTTCYGCAGGTTCAC-3′ (Staay, 2000). Oligonucleotides were obtained from Integrated DNA Technologies (San Diego, CA). The PCR conditions used were: 94 °C hot start for 2 min; 30 cycles of 30 s

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denaturation at 94 °C, annealing for 2 min at 52 °C, and elongations at 72 °C for 3 min; a final elongation at 72 °C for 10 min. The PCR product was verified through agarose gel electrophoresis and through the absence of amplification in DNA-free controls. The PCR product was cloned using the TOPO TA Cloning Kit (Invitrogen). Using the T3 and T7 primers provided by the kit, the cloned fragments were sequenced in both directions. Sequencing services were provided by SeqXcel Inc (San Diego, CA). Sequences were assembled and checked using the software Sequencher 2.0 (Gene Codes Corporation).

2.4 16S rRNA amplification, cloning and sequencing

DNA from Set B and Set C were used to obtain 16S rRNA environmental libraries from two different sampling days. The protocol for PCR, cloning and sequencing was performed similarly to the procedure for 18S rRNA above. The forward primer for PCR was 5′-AGAGTTTGATCCTGGCTCAG-3′ and the reverse was 5′-TACGGYTACCTTGTTACGACTT-3′ (Lane, 1991). The PCR conditions were: 94 °C hot start for 3 min; 28 cycles of 45 s denaturation at 94 °C, annealing for 2 min at 54 °C, and elongations at 72 °C for 3 min; a final elongation was run at 72 °C for 10 min.

2.5 Culture dependent air sampling and DNA isolation

Set D was collected by exposing *Marine Agar 2216* (Difco Laboratories Inc.) plates to the atmosphere on the rooftop of the SIO Pier pump house for 0, 5, 10, 20, and 40 min; the time exposures were done in duplicates, yielding a total of 10 plates. One set of plates was incubated at room temperature for 4 days while the duplicate set was first heat treated at 55 °C for 30 min then incubated at room temperature for 4 days. Heat treated plates served to investigate microbes found in heat resistant forms (e.g. endospores). Subsequent analysis involved similar treatment for both sets of plates. A total of eleven bacterial colonies were picked, three of which were from heat treated plates. Colonies were directly inoculated in Marine 2216 broth, incubated overnight,

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and DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN). The PCR for these samples was conducted as described in the culture independent experiments above. Additionally, two fungal colonies were picked, resuspended in TE buffer, and boiled for 5 min to lyse the cells; the boiled suspension was subjected to PCR using the 18S region probes. The PCR product was purified using QIAquick PCR Purification Kit (QIAGEN) and sequenced.

2.6 Phylogenetic analysis

DNA sequences from each sample were used to obtain the top sequence match from GenBank. ClustalW was used to construct sequence alignments with all 18S rRNA or 16S rRNA data. The alignments were analyzed using PAUP 4.0 (Swofford, 2003) to generate distance neighbor joining trees with an HKY85 nucleotide model and accompanying bootstrap values. The 18S rRNA tree was built using a region of 1.138 nucleotides and 1,339 nucleotides for the 16S rRNA tree. Phylogenetic trees were used to determine how closely related microorganisms were within our samples.

3 Results

From a total of 14 clones analyzed through a culture independent method, 18S rRNA phylogenetic analysis from sample Set A shows the presence of three phyla: Ascomycota and Basidiomycota from the Fungi kingdom, and Chlorophyta from the Viridiplantae kingdom (Fig 2). Basidiomycota was the most well represented phyla with samples closely related to the genera *Rickenella, Ceratobasidium, Tricholoma, Hyphodontia, Systotrema, Cryptococcus*, and *Malassezia*. Consistent with the air samples obtained through the MOUDI, air sampling methods using *Marine 2216* plate media revealed the presence of Basidiomycota and Ascomycota (Clones DF1 and DF2 in Fig. 2), although these were not similar to clones found in the 18S rRNA library.

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Phylogenetic analysis of air sample Set B reveals the presence of two major phyla, Firmicutes and Proteobacteria (Fig. 3). The Firmicutes were detected in nine out of sixteen 16S rRNA environmental clones (Fig. 3). *Bacillus*, *Geobacillus*, and *Paenibacillus,* three related genera, were detected among the Firmicutes. Proteobacteria were detected in seven out of sixteen 16S rRNA environmental clones (Fig. 3). *Rastolnia* was the only genus detected among the Proteobacteria. Consistent with Set B, a second air sample collected on a subsequent day (Set C) reveals the presence of Firmicutes and Proteobacteria (Fig. 3). In Set C, the Firmicutes were present in three out of twelve 16S rRNA environmental clones and the remaining nine clones belonged to the Proteobacteria phyla. *Bacillus* and *Geobacillus* were present among the Firmicutes; *Brevundimonas, Roseococcus,* and *Ralstonia* were found among the Proteobacteria (Fig. 3). Sequences highly related to that of *Ralstonia pickettii* were common in air samples Set B and Set C (Fig. 3). To minimize the number of repeats in the 16S rRNA distance tree, one sequence was selected to represent all 14 sequences closely related to *R. pickettii*. All of the represented sequences showed little sequence diversity and had a sequence match of ≥99% identity to *R. pickettii*. Although we did not detect amplification in our DNA controls, *R. pickettii* is known to be found in ultrapure water systems (Kulakov, 2002) so we believe the presence of *R. pickettii* in bioaerosols should be treated with caution.

In a culture dependent manner, aerosol samples from the SIO Pier were obtained by using *Marine Agar 2216* media (Difco Laboratories Inc.). A total of 11 bacterial colonies were analyzed revealing the presence of Firmicutes, Actinobacteria, and Proteobacteria (Fig. 3). The Firmicutes were present in six of the eleven bacterial isolates with samples of the *Bacillus* and *Oceanobacillus* genera; two Actinobacteria were isolated with samples related to the *Streptomyces* and *Micrococcus* genera; one Proteobacteria was isolated, which belongs to the *Paracoccus* genus (Fig. 3).

From the culture dependent samples, the detected fungi and bacteria were viable and grew to produce colonies. However, colonies could have developed from vegetative cells or through spore germination. Our heat treated plates inhibited all fungal

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growth but did show the eventual growth of some spore forming bacteria (clones: D4, D5, D7). *Bacillus* and *Streptomyces* species were those found in heat resistant forms.

Many of the observed fungal and bacterial sequences are from taxa commonly found in soils and other terrestrial sources. However, a few of these identified taxa are likely of marine origin. *Pycnococcus sp.* is a marine microalgae (Guillard, 1991) that is widely distributed in the Atlantic and Pacific Ocean (Campbell, 1994). *Aerobasidium pullulans* (Gao, 2009; Chi, 2007) and *Candida austromarina* (Fell and Hunter, 1974) are marine yeasts commonly found in marine environments. Studies of marine environments have found many species related to *B. subtilis, B. pumilus*, and *Paenibacillus* (Siefert, 2000). Many other species have been isolated from deep sea sediments such as marine *Streptomyces* species (Prieto-Davó, 2008), *Oceanobacillus iheyensis* (Takami, 2002), *Geobacillus stearothermophilus* and *Geobacillus kaustophilus* (Takami, 2004). *R. pickettii* has also been found among coral microbiota (Brück et al. 2007).

Air mass back trajectories were analyzed after the fact for the days of the different samples (Fig. 1). Samples A and B were obtained on days when the air mass over the SIO pier was clearly of oceanic origin. Nevertheless, this corresponded to samples showing diverse *Bacillus* sp. in the 16S rRNA libraries (see Fig. 3).

4 Discussion

Our 18S rRNA results indicate the presence of fungi as the primary eukaryotic organism in bioaerosols collected from the SIO Pier with Basidiomycota being the dominant phyla followed by Ascomycota. Only one sequence appeared outside the fungal kingdom, *Pycnococcus* sp. from the Viridiplantae kingdom. These results are similar to previous studies conducted in tropical rain forests regions and are expected based on estimates indicating that spore discharges from Ascomycota and Basidiomycota are the primary components of bioaerosols in the 1–10 µm size range (Elbert, 2006; Després, 2007). Ascomycota and Basidiomycota have also been found in cloud water by Amato et al. (2005, 2007), suggesting their potential involvement in cloud

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droplet formation as well. Most of the fungal genera we found, including *Tricholoma*, *Hyphodontia*, *Sistotrema*, *Cryptococcus*, *Aurobasidium*, and *Candida*, were found in a recent year-long study conducted over a continental air mass in Germany (Fröhlich-Nowoisky, 2009). Despite this, our fungal 18S rRNA sequences were not particularly close at the species level to those seen previously. This highlights the general presence but likely high diversity of Ascomycota and Basidiomycota in the atmosphere.

The 16S rRNA results show that Firmicutes and Proteobacteria were the predominant bacteria detected in our samples while Actinobacteria and other microbes were present at lower frequencies. These results are in agreement with studies that have found these bacterial phyla in air over urban, rural, and high alpine locations (Després, 2007). Studies on cloud water identified *Streptomyces, Micrococcus, Bacillus, Paenibacillus* and many Proteobacteria (Amato, 2005, 2007); we also found 16S rRNA sequences from these genera. The abundance of specific microbes in the air is probably influenced by the microbial distributions in surrounding environments, the specific microbial characteristics that might facilitate emission into the atmosphere, and the mechanisms of aerosol formation that are at play, and of course sampling and analysis techniques could also cause a bias in relative abundance of specific microbes. Nevertheless, there seems to be a ubiquity of specific bacteria across different atmospheric environments particularly *Bacillus* related genera.

The common presence of fungi and Bacillus-like bacterial species suggests that spores or spore forming microorganisms might be regularly more abundant in the atmosphere. Sporogenesis is well known to add to the dynamics of these ubiquitous organisms allowing them to better deal with environmental stress and probably to cycle between land, air, and aquatic environments. In our study we found a high number of Firmicutes and one *Streptomyces* related species. Endospore formation is specific to some Firmicutes, including species in the genus *Bacillus* (Onyenwoke, 2004) and some Actinobacteria (e.g., *Streptomyces*) also produce spore structures (Hardisson and Manzanal, 1976). Although there is an important correlation between spores and aerosols, it is critical to mention that the presence of non-spore forming bacteria (*M.*

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xinjiangensis, *Paracoccus* sp., *Brevundimonas* sp., *R. suduntuyensis*, and *R. pickettii*) in our samples and other studies indicates that spores are not essential for air transit.

Studies have shown fungi and bacteria to have efficient ice nucleation activity and to possibly act as cloud condensation nuclei. However, few specific species of fungi with IN and CCN properties have been identified. The *Fusarium* genus, a member of the Ascomycota, is the most well known fungus that participates in ice nucleation (Humphreys, 2001). The source from which ice nucleation arises in fungi is not well known. In bacteria, ice nucleation likely arises from a surface protein. Species with this property include members of the *Erwinia, Pseudomonas*, and *Xanthomonas* genera (Edwards, 1994). In our samples, we did not find any fungi or bacteria well known for being efficient IN or CCN, but at the same time our samples were from a unique land/air/ocean interface. Many studies on ice nucleation have focused on the activities of vegetative cells. It would be interesting to investigate how efficient our microbial spores serve as IN and CCN in contrast to their non-spore counterparts.

Contrary to our expectations that waves and bubble bursting at the sea-air interface would lead to PBA dominated by planktonic marine bacteria and phytoplankton during conditions where air masses originated over the ocean (verified by air mass back trajectories), we did not find these dominating the biotic particles of our coastal sampling site. Common marine bacterial taxa (*Pelagibacter, Roseobacter, Synechococcus*, etc.) including those commonly found in marine waters near the SIO pier (Mayali, 2010) were not found among our samples. Although we detected a *Pycnococcus*-related species, no other members of the eukaryotic ultraplankton such as diatoms or dinoflagellates were identified. This suggests that at least on the days and atmospheric conditions we sampled, marine planktonic bacteria and phytoplankton were not abundant in the air. However, such marine microorganisms might be present under different conditions (e.g. perhaps during a phytoplankton bloom).

One explanation for the lack of planktonic marine microbes but the finding of marine fungi and *Bacillus*-like species is that local beaches, with regular wetting, drying, and wave action may serve as a source of bioaerosols to coastal environments including our

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monitoring site. Since sandy beaches are ubiquitous in the world, they may represent an unappreciated source of global bioaerosol production.

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Table 1. Summary of Sampling Conditions at the SIO Pier.

Library ID	Control	Set A	Set B	Set C	Set D
Date (Pacific Time)	N/A	19 Dec 2007(13:00) to 19 Dec 2007(19:00)	18 Dec 2007(18:30) to 19 Dec 2007(13:00)	22 Dec 2007(11:30) to 22 Dec 2007(16:30)	18 Jun 2008 (15:05 to 15:45)
Sampling rate	N/A	31 lpm*	48 lpm	30 lpm	N/A
Nominal Size distribution	N/A	5.6–10 µm	0.32–3.16 µm	0.18–0.56 µm	N/A
Collection method	N/A	Cascade Impactor	Cascade Impactor	Cascade Impactor	Plate Exposure
DNA yield or colony	2ng	35 ng	543 ng	475 ng	11(3**) Colonies
Sequence analysis	N/A	18S	16S	16S	16S, 18S
Average Wind Direction	N/A	S	SSE	WNW	N
Average Wind Speed (m/s)	N/A	2.5	2.4	3	1.64
Peak Wind Speed (m/s)	N/A	5.54	6.84	5.81	3.9
Air Mass Back Trajectory	N/A	Oceanic	Oceanic	Continental	Oceanic and Continental

* lpm: liters of air per minute.

** Analyzed from heat treated plates.

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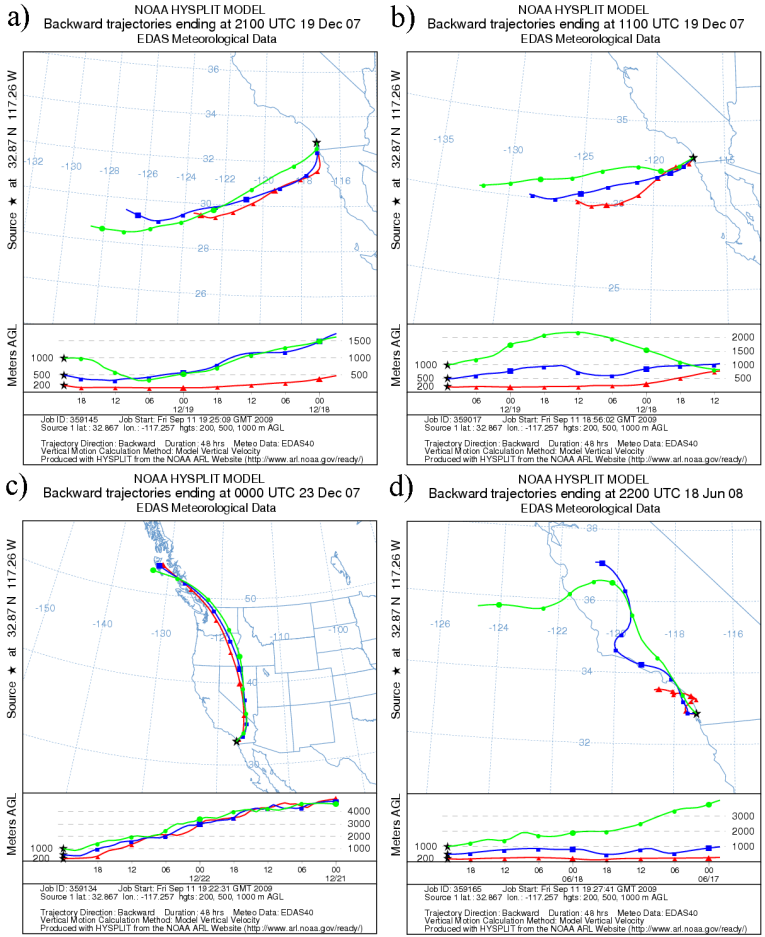

Fig. 1. Representative 48-h HYSPLIT air mass back trajectories for each filter set **(a–d)**. Dots correspond to 6 h increments and lines correspond to trajectories taken at 1000 m (green line), 500 m (blue line), and 200 m (red line).

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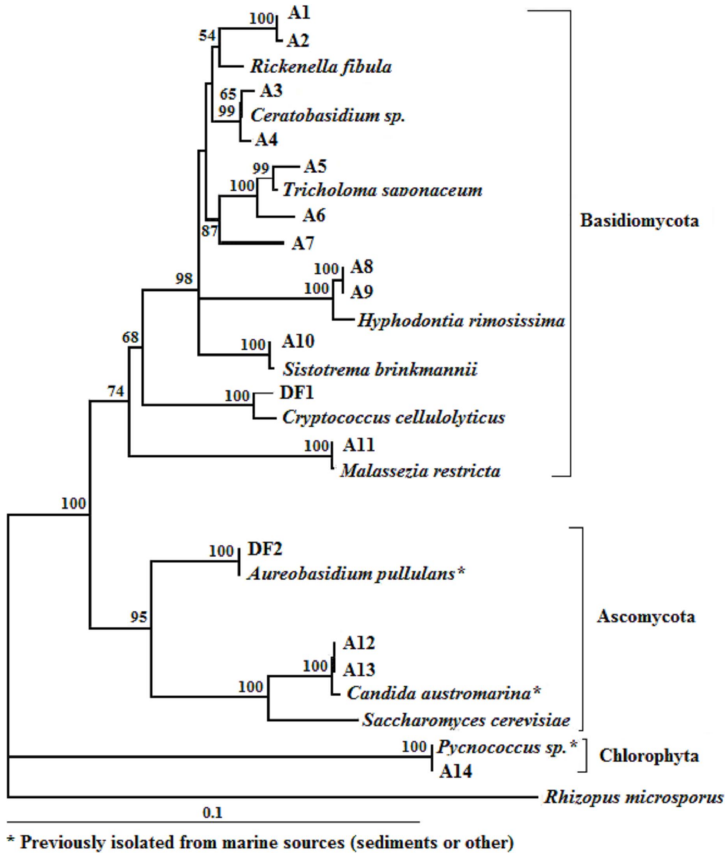

Fig. 2. A neighbor joining tree shows the phylogenetic relationships among our 18S rRNA environmental clones, plate isolates, and their top sequence matches obtained through NCBI BLAST. The distance tree is based on a sequence alignment spanning over a 1,138 nucleotide region of the 18S rRNA gene and is rooted with *R. microsporus*. The distances were calculated using the HKY85 nucleotide model. The bootstrap analysis was done using 100 replicates.

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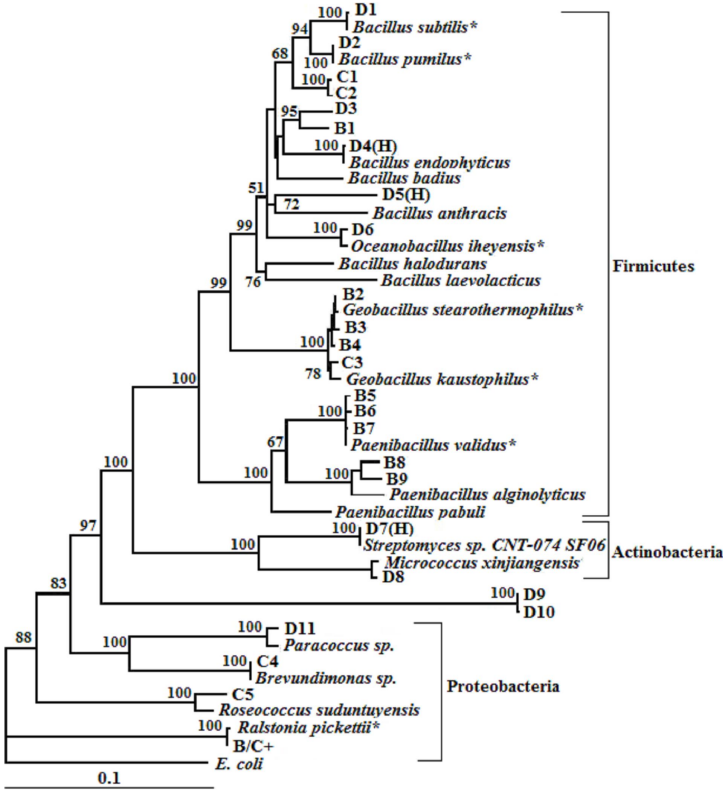

Fig. 3. A neighbor joining tree shows the phylogenetic relationships among our environmental clone library sequences, plate colony sequences, and their top sequence matches obtained through NCBI BLAST. The distance tree is based on a sequence alignment spanning over a 1,339 nucleotide region of the 16S rRNA gene and is rooted with *Escherichia coli*. The distances were calculated using the HKY85 nucleotide model. The bootstrap analysis was done using 100 replicates.

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Detection and phylogenetic analysis of coastal bioaerosols

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