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PRELIMINARY PCR-TTGE ANALYSES OF BACTERIAL COMMUNITIES ASSOCIATED WITH POLLEN FROM ANEMOPHILOUS TREES: POTENTIAL IMPACTS ON PLANTS AND HUMAN HEALTH

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ABSTRACT
Pollen from wind-pollinated plants is the predominant cause of polinosis which corresponds mainly to allergic rhinitis and conjunctivitis. Bacterial communities associated to leaves and roots surface were analysed for many years with both culture-dependent and independent methods. However, microbiota of pollen grains was rarely investigated and never with a molecular fingerprint technique. The aim of this study was to perform a preliminary analysis of the pollen microbiota by applying PCR-TTGE method. Pollen samples were collected from various anemophilous trees genera (Cephalotauxus, Cupressus, Pinus, Platanaus and Quercus) in Montpellier (France) in 2013. Thorough pollen preparation was essential to a successful recovery of DNA from pollen. Regarding the results, pollen microbiota was tree genus-dependent. In addition, intra-tree genus variations were also observed. The Gamma proteobacteria class was the most represented in pollen samples whatever the tree genus. Among this class, some bacteria were recognized as phytopathogens (Pseudomonas, Erwinia) but also opportunistic human pathogens (Pseudomonas, some enterobacteria). PCR-TTGE is a suitable tool to analyse the microbiota associated with pollen responsible for phytopathogenicity or affecting the human respiratory tract.

Keywords: polinosis; anemophilous trees; pollen microbiota; health; PCR-TTGE

INTRODUCTION
Pollen grains represent a category of Primary Biological Aerosol Particles (PBAP) in addition to bacteria, virus, fungal spore, algae, cyanobacteria (Després et al., 2012). During pollination, pollen grains come into contact with human respiratory or conjunctival mucosa and could be responsible for pollinosis which correspond mainly to allergic rhinitis and conjunctivitis. In France, pollinosis affect from 10 to 15% of the general population and are increasing in urban areas (Ravault et al., 2005). In some cases, small pollen grains can induce asthma attacks (Heydenreich et al., 2012). Allergic rhinitis triggered by the pollen grains of some seasonal plants is commonly named "hay fever", and appears mostly during haying season. However, atopic patients with seasonal allergies present attacks (Vanneste et al., 2007; Yalcin et al., 2015) and less frequently during haying season. However, atopic patients with seasonal allergies present hay fever throughout the year. The pollen grains that provoke hay fever change between individuals and from region to region. The hardly visible pollen of wind-pollinated plants such as trees, grasses, and weeds are yet the predominant cause (D’Amato et al., 2007). In Mediterranean region, Piniaceae and mostly Cupressaceae pollen grains constitute most clinically relevant trees pollen (D’Amato et al., 2007; Yalced et al., 2013). Moreover, pollen is regarded as a source of not only allergens but also immunomodulatory molecules, which present major roles in sensitization and/or the exacerbation of allergies (Kamiyo et al., 2009). Recently, nanovesicles containing allergens (named pollensomes) released during in vitro germination of pollen grains were described to contribute to allergic reaction (Prado et al., 2015).

For many years, bacterial communities associated to plants were analysed particularly root-inhabiting (Buée et al., 2009; Bulgarelli et al., 2012), leaf-surface inhabiting (Yashiro et al., 2011; Izhaki et al., 2013) and less frequently flower-inhabiting (Alekket et al., 2014) bacterial microbiota. Both culture-dependent and independent methods were performed to explore bacterial microbiota. Since the diameter of pollen grains from anemophilous species varies from 17 to 58 μm (Després et al., 2012), pollen grains may represent a support for bacteria. Moreover, many exuded compounds of pollen grains are known to be attractive for bacterial colonizers (Alekket et al., 2014).

Bacterial communities associated to pollen called bacterial pollen microbiota were rarely investigated. Colldahl and Carlsson (1968) isolated the Gram-negative Pseudomonas maltophilia (currently Stenotrophomonas maltophilia) from pollen samples. The presence of microorganisms on surface of pollen was then confirmed by scanning electron microscopy (Colldahl and Nilsson, 1973). Later, Spiewak et al. (1996a) highlighted the presence of a mixed microflora consisted of Gram-positive and Gram-negative mesophilic bacteria, thermophilic actinomycetes and fungi on allergenic pollen grains. Moreover, Spiewak et al. (1996b) showed that Gram negative bacteria such as Pantoea agglomerans endotoxin associated to pollen should be considered as a potential factor aggravating pollinosis. More recently, Heydenreich et al. (2012) reported that grass pollen grains were colonized by Gram-negative bacteria as Acinetobacter Iwoffii and Gram-positive bacteria belonging to the genus Bacillus which presents adjuvant activity inducing inflammatory T cell responses. Few studies on plant protection have been carried out in order to detect bacteria responsible for plant disease as Pseudomonas syringae pv actinidiae on the kiwifruit pollen grains (Vanneste et al., 2011). All these studies were investigated with culture-dependent method based on the isolation of bacteria on different agar media. Molecular methods were never used.

Currently, bacterial communities associated with humans (McCartney, 2002; Roudriëre et al., 2009; Michon et al., 2012), animals (Richards et al., 2005; Navarreté et al., 2012), plants (Lambais et al., 2014), foods (Ogier et al., 2004; Hamdouche et al., 2015) or environment (Lyautey et al., 2005) are analysed by culture-independent approaches such as genetic fingerprinting methods (PCR-DGGE, PCR-TTGE). The 16S rRNA gene is the most frequently gene used for amplification. Separation of PCR products in TTGE is based on the decrease of the electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing DNA denaturants according to a temperature gradient. Molecules with various sequences will have a different melting behaviour and will stop migrating at distinct position in the gel (Ogier et al., 2002). The aim of this study was to perform a preliminary analysis of the bacterial communities associated with pollen isolated from plants responsible for
polinosis by applying PCR-TTGE method. A PCR anchored on the bacterial 16S rRNA gene followed by a TTGE analysis was carried out on DNA extracts from several pollen grain samples collected in Mediterranean region. On the one hand, methodological aspects were developed including a comparative study of different pollen preparation methods. On the other hand, bacterial diversity of a set of samples were analysed with a 16S rDNA based PCR-TTGE approach.

**MATERIAL AND METHODS**

**Pollen grains sampling**

Pollen samples were collected from various anemophilous trees (Tab 1) in Montpellier, France (GPS data = 43.6° N & 3.9° W) between February and April 2013, in accordance with the pollinic bulletins of the French aerobiology network RNSSA (Réseau National de Surveillance Aérobiologique). Collected pollen samples belonged to the genera *Pinus* (x = 5), *Cupressus* (x = 4), *Cephalotaxus* (x = 1), *Quercus* (x = 1), and *Platanus* (x = 1). Data concerning the samples figured in Table 1. According to the type of male inflorescence, pollens were either directly collected or spikes were cut with sterile scalpels. In both cases, pollen grains were aseptically collected in sterile storage bags after shaking and sieving through sterile gauze. Careful consideration was given to collect the pollen grains in order to avoid mixed pollen samples from different plants. Pollen samples were stored at 4°C in the dark. Quantification of bacteria in pollen presented in Table 1 was performed according to the protocol described below.

**Table 1** Data concerning the pollen sampling. Quantification of bacteria in pollen was performed according to the protocol described above.

| Sample name | Date of sampling | Plant genus | Geographic location in Montpellier city* | Enumeration number of bacteria / g pollen |
|-------------|------------------|-------------|----------------------------------------|------------------------------------------|
| P1          | 2013/04/10       | *Pinus*     | I                                      | 4.0 10^5                                 |
| P2          | 2013/04/10       | *Pinus*     | II                                     | 1.4 10^5                                 |
| P3          | 2013/04/10       | *Pinus*     | I                                      | 6.7 10^5                                 |
| P4          | 2013/04/14       | *Pinus*     | III                                    | 3.9 10^5                                 |
| P8          | 2013/04/22       | *Pinus*     | IV                                     | 1.9 10^5                                 |
| C1          | 2013/03/21       | *Cupressus* | I                                      | 1.4 10^5                                 |
| C1b         | 2013/03/21       | *Cupressus* | I                                      | 5.4 10^5                                 |
| C2          | 2013/02/23       | *Cupressus* | V                                      | 2.0 10^5                                 |
| C3          | 2013/03/25       | *Cupressus* | I                                      | 5.7 10^5                                 |
| Cx          | 2013/04/03       | *Cephalotaxus* | VI                                    | 5.0 10^5                                 |
| Q           | 2013/04/18       | *Quercus*   | VI                                     | 7.0 10^5                                 |
| P1a2        | 2013/04/22       | *Platanus*  | VII                                    | 5.7 10^5                                 |

Legend: * I- Faculty of Sciences campus, II- Faculty of arts campus, III- campus, VI- Garden of plants, VII- Albert 1st square.

**Preparation of pollen samples**

Pollen samples were prepared by five different protocols (A, B, C, C.adn, C.pca and C.tsh) before DNA extraction (Fig 1). The primary objective was to highlight the best protocol to extract effectively all bacterial DNA associated to pollen grains. For protocol A, 30 mg of pollen sample was suspended directly in 150 μl of Tris-EDTA (TE) buffer with 1 μl of lysozyme (Sigma) and incubated at 37 °C for 18 h. For protocol B, 30 mg of pollen sample were centrifuged (12,000 g for 10 min) thrice in 150 μl of TE buffer. Then, the final pellet was suspended in 150 μl of TE buffer with 1 μl of lysozyme and incubated at 37 °C for 18 h. The protocol C was based on the method described by Vanneste et al. (2011) including few modifications as follows: 30 mg of dry pollen suspended in 1 ml of sterile distilled water were sonicated for 5 min. Suspension was shaken with a rotary shaker for 60 min at 120 rpm. After settling, the supernatant was filtered through sterile gauze and centrifuged at 10,000 g for 20 min at 6°C. The supernatant was discarded and the pellet was rehydrated with 1 ml of sterile distilled water. This solution is referred to as the Final Concentrate (FC). From this FC, three different variations to protocol C called C.adn, C.pca and C.tsh were performed in order to optimise the recovery of DNA. For C.adn, DNA was directly extracted from 500 μl of FC. For C.pca, 100 μl of the FC were spread onto Plate Count Agar (PCA) (Bio-Mérieux) and incubated at 30 °C for 48 h. For C.tsh, 100 μl of the FC were spread onto Tryptic Soy Agar supplemented with 5 % of horse blood (TSH) (Bio-Mérieux) and incubated at 30 °C for 48 h. For C.pca and C.tsh, DNA was extracted from cultures collected at the surface of PCA or TSH plates respectively. The last step of protocol C consists of re-suspension in 150 μl of TE buffer with 1 μl of lysozyme and incubation at 37 °C for 18 h.

**Bacterial enumeration**

For protocol C, three decimal dilutions were carried out in saline serum from Final Concentrate (FC). Then 100 μl of FC and each dilution were plated in duplicate on PCA agar and incubated at 30 °C for 18 h.

**DNA extraction**

DNA was extracted from the five previous protocols of pollen preparation, following the recommendations of the MasterPure™ DNA purification Kit (Epicentre). Quality of DNA extracts was evaluated using UV spectrophotometry (rate 260 nm / 280 nm) and diluted to obtain a final concentration of 50 μg/ml.

**PCR –Temporal Temperature Gradient Gel Electrophoresis (TTGE) analysis**

A 199 bp-fragment (from position 338 to position 536, *Escherichia coli* numbering) overlapping the V3 variable region of 16S rDNA (position 338 to position 534, *E. coli* numbering) (Sundquist et al., 2007) was amplified using primers HDA1F-GC and HDA2 (Oger et al., 2002). A 40 bp GC-clamp was added to the forward primer. PCR was performed in a final volume of 50 μl containing 200 μM each dNTP (Fermentas), 200 mm each primer (Sigma), 2.5 U of Fast Start Taq DNA Polymerase (Roche) in the appropriate 1x reaction buffer with 1.8 mM MgCl2 and 1 μl of the DNA extract. Amplification program was carried out as follows: an initial denaturation step at 95 °C for 2 min followed by 30 cycles of 95 °C for 1 min, 62 °C for 30 s, 72 °C for 1 min and 72 °C for 7 min for the final extension. PCR products were checked using conventional electrophoresis in 1.5 % (w/v) agarose gel with 1x Tris Borate EDTA (TBE) buffer and then submitted to TTGE analysis using a Dcode Universal Mutation Detection System (Bio-Rad). PCR products (1 μl) were loaded into 8% (w/v) agarose (37.5:1), 7 M urea, 40 μl TEMED and 0.1 % (w/v) ammonium persulfate gels. Migrations were performed in 1x Tris Acetate EDTA (TAE) buffer with additional magnetic shaking in the electrophoresis compartment. A pre-migration for 15 min at 63 °C and 20 V was followed by migration for 16 h at 46 V with an initial temperature of 63 °C and a final temperature of 70 °C corresponding to an increase of 0.4 °C / h. Gels were stained for 15 min with 0.5 mg / ml ethidium bromide in 1x TAE buffer, rinsed for 45 min in 1x TAE buffer and then photographed on a UV transilluminator.
TTGE bands analysis and sequencing

Dominant bands were cut from TTGE gels, rinsed twice with molecular biology grade water and eluted overnight in 10 mM Tris buffer (pH 8.5) at 37 °C. Extracted DNA was re-amplified using primers HDA1 and HDA2 without GC-clamp as previously described (Michon et al., 2010). Then PCR products were sequencing on an applied automatic sequencer (Cogenics) by using the forward primer HDA1. DNA sequences were visualized and analysed with BioEdit program version 7.0.9 (Hall, 1999). The 16S rRNA gene sequences were screened using GenBank’s Blast program (Altschul et al., 1990).

Fingerprinting and statistical analysis

The TTGE gel images were analyzed using Image Quant TL software v. 2003 (Amersham Biosciences). Individual lanes of gel images were aligned which permits detection and record of the relative position of each DNA band. TTGE patterns were manually scored by presence and absence of co-migrating bands between lanes. Pairwise community similarities were quantified using the Dice similarity coefficient (SD): SD = 2 A b / (A a + B b) (Heyndrickx et al., 1996) where A a represented the number of bands detected in sample A, B b represented the number of bands in sample B, and N c represented the number of bands common to both samples. A cluster analysis was carried out using the similarity matrix to group pollen samples according to their similarity index. A Principal Component Analysis (PCA) was also used as a multivariate technique for exploratory data analysis using Statistica (version 7) software (StatSoft, USA).

RESULTS

Bacterial communities were analysed on pollen samples from various wind-pollinated trees in order to evaluate the microbiota associated to pollen. The comparison of microbial ecology of pollen grains from various anemophilous trees was performed by PCR-TTGE analysis.

Optimization of preparation of pollen samples and bacterial numeration

Five protocols were evaluated to prepare the pollen samples. Figure 2 shows TTGE patterns obtained from pollen samples of five Pinus specimens, i.e., P1-4 and P8 (Tab 1) prepared according to four different protocols (A, C.adn, C.pca and C.tsh).

For P8 sample, one band was detected for protocols A and C.adn while 16 bands were detected for protocols C.pca and C.tsh. Thus, both protocols C.pca and C.tsh highlight the highest number of detected bands whatever the sample. The cluster analysis of TTGE patterns obtained from pollen samples of the five Pinus specimens prepared according to four protocols showed two main dissimilar clusters: the first cluster group patterns obtained from protocols C.pca and C.tsh while the second cluster comprised patterns obtained from protocols A and C.adn (Fig 4).

The Principal Component Analysis performed on the same TTGE patterns showed 70% of the variability of samples according to four protocols with or without a step of culture before DNA extraction (Fig 5).
Two distinct groups could be discriminated: the first group contained samples obtained from protocols with step culture (C.pca and C.tsh), a second group included samples prepared without step culture (protocols A and Cadn). Bacterial enumeration from PCA medium varied from 1.4 x 10^3 to 4.0 x 10^4 CFU / g pollen and from 2.0 x 10^2 to 5.7 x 10^6 CFU / g pollen for Pinus and Cupressus, samples, respectively (Table 1). Bacterial enumeration ranged from 7.0 x 10^1 to 5.7 x 10^6 CFU / g pollen for Quercus, Cephalotaxus and Platanius.

Bacterial communities associated to pollen samples

The objective of this study was to evaluate the bacterial communities associated to pollen grains from different tree genera, i.e., Pinus, Cupressus, Cephalotaxus, Quercus, and Platanius using PCR-TTGE. Concerning pollen samples from Pinus, a total of 53 DNA bands were observed in total of the samples (Fig 2). Among the 53 observed bands, 46 bands were cut and sequenced. DNA sequences obtained from cut bands varied from 147 to 172 bp compared to the 199 bp of the V3 region. First, we demonstrated that several sequences corresponded to chloroplast DNA. Presence of chloroplast DNA was not unexpected because of the high similarity between bacterial and chloroplast 16S rRNA gene sequences in the context of endosymbiotic origin of chloroplast (McFadden 2001). As a result, bacterial but also chloroplast 16S rRNA genes could be amplified with primers used in this study. Then, we detected a majority of bacteria belonging to the Enterobacteriaceae family such as Erwinia sp., Pantoaea sp., Enterobacter sp., Klebsiella sp., and Providencia sp. but also to Pseudomonadaeae family such as Pseudomonas sp., and Bacillus sp. Within the genus Pinus, the five TTGE patterns corresponding to C.pca protocol were very different from one tree to another. Indeed, we observed that bacterial communities associated to pollen seemed to vary among the genus Pinus. Only bacterial strains belonging to Pseudomonas genus seemed to be present in four Pinus samples from five. Concerning pollen samples from Cupressus, Cephalotaxus, Quercus, and Platanius, a total of 99 DNA bands were observed in total of the samples which permitted to obtain 81 DNA sequences. From Cupressus samples, we found bacterial belonging to Enterobacteriaceae family such as Erwinia sp., Pantoae sp., Enterobacter sp., Klebsiella sp., and Providencia sp. but also to Pseudomonadaeae family such as Pseudomonas sp., and Bacillus sp. Within the genus Cupressus, the five TTGE patterns corresponding to C.pca protocol were very different from one tree to another. Finally, for Platanius pollen sample, the TTGE pattern comprises bacteria from Enterobacteriaceae (Tatumella sp. and Erwinia sp.), Pseudomonas sp. and Stenotrophomonas sp.

DISCUSSION

In the present study, we analysed bacterial communities associated to twelve pollen samples collected from five anemophilous tree genera at different locations in Montpellier (France). We used a molecular fingerprint technique called PCR-TTGE which was already applied to determine bacterial communities associated to pollen grains from four plant species (Spiewak et al., 2012; Navarrete et al., 2012). Five pollen preparation protocols were evaluated. Preparation of pollen samples before DNA extraction was a critical step of the PCR-TTGE analyses since it applied decisive to obtain the totality of bacterial communities associated to a sample. As concerns the number of detedected DNA bands for each TTGE profile, mean values varied according to the pollen preparation protocol. Thus, the best results were obtained with the protocol adapted from Vanneste et al. (2011) followed by a culture dependent step on a non-selective medium (PCA medium) or enriched medium (TSH medium) before DNA extraction, amplification and TTGE analyses. Indeed, the previous mentioned preparation of pollen comprising sonication and a 1-hour shaking allowed to optimally recovery bacteria from exine pollen wall. Then, the culture-dependent step permitted to concentrate bacteria. We obtained TTGE patterns with a largest number of DNA bands corresponding to main but also less abundant bacteria present on pollen samples. However potential viable but nonculturable (VBNC) bacteria could not be detected. The culture-independent protocol highlighted only the main bacteria. The bacterial enumeration of pollen samples varied from 7.0 x 10^1 to 5.7 x 10^6 CFU/g pollen. These results were concordant with those obtained by Spiewak et al. (1996a) for different trees.

The bacterial communities associated to pollen samples varied among two plant genera (inter-genus variations) but also inside a same genus (intra-genus variation). Indeed, we observed very different TTGE profiles for the five pollen samples from Pinus and for the four pollen samples from Cupressus. Bacteria belonging to the class Gammaproteobacteria (Enterobacteriaceae, Pseudomonadaeae, Xanthomonadaeae…) were the most represented in pollen samples before bacteria belonging to the phylum Firmicutes, whatever the plant genera and the pollen samples were. The sequences observed by Spiewak et al. (1996a) which highlighted in particular mixed microflora consisting of Gram-positive and Gram-negative mesophilic bacteria on allergenic pollen grains. Bacteria belonging to Gammaproteobacteria class represented 73% of the 16S rDNA sequences obtained and were detected in most of samples. These results are in accordance with those of Wetzel et al. (2010) which showed that 81.2% of bacterial species isolated from edible flowers belonged to the class Gammaproteobacteria. Hence, we demonstrated that the intragenomic and intraspecific heterogeneity of the 16S rDNA gene, it is very difficult to obtain identification at the species level and even at the genus level. It is a classical disadvantage of the PCR-TTGE method (Michon et al., 2012). Some Enterobacteriaceae species are commonly present in plant. We found DNAs of Erwinia sp. and Pantoaea sp. in Pinus and Cupressus samples. Erwinia is a well-known bacterial genus associated to plants. Some Erwinia species could lead to damages of plant structure, wilting, dieback, yellowing or rot (Thomson et al., 1999). The main phytopathogenic Erwinia species are E. amylovora and E. carotovora responsible for the fire blight of Rosaceae especially apple and pear (Piquè et al., 2015), and soft rot diseases respectively (Parent et al., 1996). Pantoaea agglomerans (formerly Erwinia herbicola) could be opportunistic pathogen in cases of weakness of the plant (Dutkiewicz et al., 2016). Dickeya sp., Enterobacter sp. and Klebsiella sp. DNAs were also detected in our pollen samples. The genus Dickeya comprises several species particularly D. chrysanthemi (formerly Erwinia chrysanthemi) known to be phytopathogenic (Toth et al., 2011). Enterobacter asburiae, Enterobacter cloacae and Enterobacter cowanii were already recovered from plants (Wetzel et al., 2010; Humann et al., 2011). E. asburiae was considered as a Plant Growth Promoting Rhizobacteria (PGPR) particularly for Citrus reticulata (Thokhom et al., 2014) whereas E. cloacae is showed to be phytopathogenic for numerous plants such as tomato (Barrar et al., 2003) and tomato and pepper (Michon et al., 2012). E. asburiae isolated from tracheal aspirate of a patient suffering from pulmonary infections should be taken into account as a potential human pathogen which was especially possible for a few cases of infections in immunocompromised patients (Leneveu-Jenvirin et al., 2013). Regarding the phylum Firmicutes which represents 14% of the 16S rDNA sequences obtained, we underscored the presence of Bacillus strains in particular Bacillus cereus which is well-known as volatile human pathogen (Bottone, 2010), and Staphylococcus pasteuri responsible for a few cases of infections in immunocompromised patients (Morfín-Otero et al., 2012). Pollen samples from Cephalotaxus, Quercus and Platanius were also Gammaproteobacteria such as Enterobacteriaceae and Pseudomonadaeae strains.

Indeed, we detected in pollen grains from anemophilous (deciduous and coniferous) trees various bacterial genera, i.e., Pseudomonas, Erwinia and Enterobacter well-known as responsible for a wild range of human diseases and plant infections.

CONCLUSION

In this present study, we analysed the first time bacterial communities associated to pollen grains from five plant genera, namely Pinus, Cupressus, Cephalotaxus, Quercus and Platanius by a molecular method. PCR-TTGE is a suitable tool since bacterial microbiota was determined for each pollen samples. Bacterial communities varied inside a plant genus and from genus to genus. We highlighted here the potential presence of phytopathogens and also human potential opportunistic bacteria on allergenic pollen grains. Indeed, pollen grains
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