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

Françoise Fons 1, Stefaniya Hantova2, Yasmine Hamdouche3, Sylvie Rapior1, and Corinne Teysnier2*3

Address(es): Dr. Corinne Teysnier.
1Université de Montpellier, UFR des Sciences Pharmaceutiques, Laboratoire de Botanique, Phytochimie et Mycologie, UMR 5175 CEFE / Equipe Substances Naturelles et Médiation Chimiques, 15 avenue Charles Flahault, 34093 Montpellier cedex 5, France.
2Université de Montpellier, UFR des Sciences Pharmaceutiques, Laboratoire de Bactériologie, Virologie et Contrôle Microbiologique, 15 avenue Charles Flahault, 34093 Montpellier cedex 5, France.
3UMR 95 QualiSud / Cirad TA B, Université de Montpellier, UFR des Sciences Pharmaceutiques, Laboratoire de Bactériologie, Virologie et Contrôle Microbiologique, 16, rue J-F. Breton, 34398 Montpellier cedex 5 (France), Phone: (33) 4 67 61 57 78.

*Corresponding author: corinne.teysnier@umontpellier.fr
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ABSTRACT

Pollen from wind-pollinated plants is the predominant cause of pollinosis 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 (Cephalotaxus, 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 Gammaproteobacteria 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).

PCRTTGE is a suitable tool to analyse the microbiota associated with pollen responsible for phytopathogenicity or affecting the human respiratory tract.

Keywords: pollinosis; 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 hay fever throughout the year. The pollen grains which 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, Pinusaceae and mostly Cupressaceae pollen grains constitute most clinically relevant trees pollen (D’Amato et al., 2007; Yalçın 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 (Kamijo et al., 2009). Recently, nanovesicles containing allergens (named pollenosomes) 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 (Bué et al., 2009; Bulgarelli et al., 2012), leaf-surface inhabiting (Yashiro et al., 2011; Izhaki et al., 2013) and less frequently flower-inhabiting (Aleklett 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 exudated compounds of pollen grains are known to be attractive for bacterial colonizers (Aleklett et al., 2014).

Bacterial communities associated to pollen called bacterial pollen microbiota were rarely investigated. Coldhald 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 (Coldhald 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 lwoffi 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 cinidium 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; RoudièrE et al., 2009; Michon et al., 2012), animals (Richards et al., 2005; Navarrete et al., 2012), plants (Lambais et al., 2014), foods (Ogiër et al., 2004; Hamdouche et al., 2015) or environment (Lyautet 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 temperature. Molecules with various sequences will have a different melting temperature.
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 RNRS (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 scalpel. 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 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 below.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Date of sampling</th>
<th>Plant genus</th>
<th>Geographic location Montpellier city*</th>
<th>Enumeration number of bacteria / g pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>2013/04/10</td>
<td><em>Pinus</em></td>
<td>I</td>
<td>4.0 (10^4)</td>
</tr>
<tr>
<td>P2</td>
<td>2013/04/10</td>
<td><em>Pinus</em></td>
<td>II</td>
<td>1.4 (10^5)</td>
</tr>
<tr>
<td>P3</td>
<td>2013/04/10</td>
<td><em>Pinus</em></td>
<td>I</td>
<td>6.7 (10^4)</td>
</tr>
<tr>
<td>P4</td>
<td>2013/04/14</td>
<td><em>Pinus</em></td>
<td>III</td>
<td>3.9 (10^5)</td>
</tr>
<tr>
<td>P8</td>
<td>2013/04/22</td>
<td><em>Pinus</em></td>
<td>IV</td>
<td>1.9 (10^5)</td>
</tr>
<tr>
<td>C1</td>
<td>2013/03/21</td>
<td><em>Cupressus</em></td>
<td>I</td>
<td>1.4 (10^5)</td>
</tr>
<tr>
<td>C1b</td>
<td>2013/03/21</td>
<td><em>Cupressus</em></td>
<td>I</td>
<td>5.4 (10^5)</td>
</tr>
<tr>
<td>C2</td>
<td>2013/02/23</td>
<td><em>Cupressus</em></td>
<td>V</td>
<td>2.0 (10^4)</td>
</tr>
<tr>
<td>C3</td>
<td>2013/03/25</td>
<td><em>Cupressus</em></td>
<td>I</td>
<td>5.7 (10^4)</td>
</tr>
<tr>
<td>Cx</td>
<td>2013/04/03</td>
<td><em>Cephalotaxus</em></td>
<td>VI</td>
<td>5.0 (10^4)</td>
</tr>
<tr>
<td>Q</td>
<td>2013/04/18</td>
<td><em>Quercus</em></td>
<td>VI</td>
<td>7.0 (10^5)</td>
</tr>
<tr>
<td>Pla2</td>
<td>2013/04/22</td>
<td><em>Platanus</em></td>
<td>VII</td>
<td>5.7 (10^2)</td>
</tr>
</tbody>
</table>

Legend: * I: Faculty of Sciences campus, **II**: Faculty of arts campus, **III**: Faculty of Pharmacy campus, **IV**: Garden of plants, **V**: Albert 1st square.

**Preparation of pollen samples**

Pollen samples were prepared by five different protocols (A, B, C.adn, C.pca and C.tsh) before DNA extraction (Fig 1).

**Figure 1** Presentation of the different protocols used to prepare pollen samples before DNA extraction. For protocols C.pca and C.tsh, an additional culture on PCA medium and TSH medium were performed respectively before extraction with the MasterPure™ DNA purification Kit (Epicerent).

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 (Epicerent). 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 (Ogger 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 nm each primer (Sigma), 2.5 U of Fast Start Taq DNA Polymerase (Roche) in the appropriate 1x reaction buffer containing 2 μM 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 TBE buffer in the appropriate 1x reaction 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) bisacrylamide (37:5:1), 7 M urea, 40 μl TEMED and 0.1 % (w/v) ammonium persulphate 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 $S_D = \frac{2 N_c}{N_a + N_b}$ (Heyndrickx et al., 1996) where $N_a$ represented the number of bands in sample A, $N_b$ 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 grouped patterns obtained from protocols C.pca and C.tsh and 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 Cadv). Bacterial enumeration from PCA medium varied from 1.4 $10^5$ to 4.0 $10^5$ CFU/g pollen and from 2.0 $10^5$ to 5.7 $10^5$ CFU/g pollen for Pinus and Cupressus, samples, respectively (Table 1). Bacterial numeration ranged from 7.0 $10^5$ to 5.7 $10^5$ CFU/g pollen for Quercus, Cephalotaxus and Platanus.

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 Platanus 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 sequence 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 Pseudomonadaceae family such as Pantoea agglomerans (formerly Erwinia herbicola) which could be opportunistic pathogen in cases of weakness of the plant (Dutkiewicz et al., 2016). Dickeya sp., Enterobacter sp. and Klebsiella sp. DNA bands were also detected in our pollen samples. The genus Dickeya comprises several species particularly D. chrysanthemi (formerly Erwinia chrysanthemi) known to be phytopathogens (Toth et al., 2011).

Concerning pollen samples from Cupressus, Cephalotaxus, Quercus, and Platanus, 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 bacteria belonging to Enterobacteriaceae family such as Erwinia sp., Pantoa sp., Trabulsiella sp., and Providencia sp. but also to Pseudomonadaceae family such as Pantoea agglomerans (formerly Erwinia herbicola) which is well known bacterial genus associated to plants. Some bacteria belonging to Pseudomonas genus seemed to be present in four Pinus samples from five families.

Bacterial 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 Wetzet al. (2010) which showed that 81.2% of bacterial species isolated from edible flowers belonged to the class Gamma-proteobacteria. Moreover, 75% of the sequences were from 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 Pantoea 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 (Piqué et al., 2015), and soft rot diseases respectively (Parent et al., 1996).

Enterobacteriaceae is a well-known family of bacteria which constitute human intestinal microbiota but several species or serotypes can also be pathogenic to human. Most of them are recognized as opportunistic pathogen and causes respiratory symptoms. In particular, Pantoaea strains are responsible for respiratory infections in immunocompromised patients (Flores Popoca et al., 2012; Kursun et al., 2012; Walterson and Stravrinides, 2015; Dutkiewicz et al., 2016). Enterobacteriaceae family is the second most common in sputa and from respiratory tract of cystic fibrosis patients (Winstanley et al., 2016). In addition, P. mosselii should be taken into account as a potential human pathogen which was especially isolated from tracheal aspirate of a patient suffering from pulmonary infections (Leneveu-Jenvin 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 Platanus were also Gammaproteobacteria such as Enterobacteriaceae and Pseudomonadaceae 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 wide range of human diseases and plant infections.

CONCLUSION

This study has contributed to our understanding of bacterial communities associated with pollen grains collected from Pinus, Cupressus, Quercus and Platanus. Three bacterial communities were identified: the first group predominantly composed of bacterial genera belonging to the Enterobacteriaceae family such as Erwinia, Pantoaea, Providencia and Pantoea, the second group consisting of bacterial genera belonging to the Pseudomonadaceae family such as Pantoea agglomerans and the third group comprising rare and species-specific bacterial genera. Our study demonstrated that bacterial diversity in pollen is high and that this diversity could be useful for forensic applications. Moreover, our study highlighted the potential of pollen as a source of bacterial species which could be of interest for forensic applications.

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