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Analysis of bacterial communities on aging flue-cured tobacco leaves by 16S rDNA PCR–DGGE technology

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Abstract Many microorganisms, growing on aging flue-cured tobacco leaves, play a part in its fermentation process. These microflora were identified and described by culture-dependent methods earlier. In this study we report the identity of the microflora growing on the tobacco leaf surface by employing culture-independent methods. We have amplified microbial 16S rDNA sequences directly from the leaf surface and used denaturing gradient gel electrophoresis (DGGE) to identify bacterial community on the tobacco leaves. Our culture-independent methods for the study of microbial community on tobacco leaves showed that microbial community structures on leaves of variety Zhongyan 100, NC89 and Zhongyan 101 were similar between 0 and 6 months aging, and between 9 and 12 months aging, while the similarity is low between 0 and 6, and between 9 and 12 months aging, respectively. There were certain similarities of bacterial communities (similarity up to 63%) among the three tobacco varieties for 0 to 6 months aging. Five dominant 16S rDNA DGGE bands A, B, C, D and E were isolated, cloned, and sequenced. They were most similar to two cultured microbial species *Bacteriovorax* sp. EPC3, *Bacillus megaterium*, and three uncultured microbial species, respectively.

Keywords Flue-cured tobacco · Fermentation · 16S rDNA · PCR–DGGE

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Introduction

The most important type of tobacco in China and USA is the flue-cured tobacco. Cured but unaged tobacco is unsuitable for cigarette products because it has a sharp, disagreeable odor and an undesirable aroma, and produces irritating smoke with unacceptably harsh flavor. To improve these conditions, flue-cured tobacco, similar to Burley, Maryland and Turkish tobacco, is subjected to a further process called aging or fermentation. Aging greatly improves the aroma and other qualities desirable in smoking products. The aging process can be natural or forced, depending upon time, temperature, and humidity (Tso 1979).

The mechanism of flue-cured tobacco fermentation, especially the effect of microorganisms, was not determined. It was reported that the number of microbes per gram of tobacco was very small at the start of aging, and declined gradually along with aging; the species of organisms identified on the flue-cured leaves were those commonly found in soil and air (Dixon et al. 1936). The small quantities of enzymes found in some samples appeared to decrease with the progress of aging. Dixon et al. 1936 concluded that the contribution of both microorganisms and enzymes to the aging of flue-cured tobacco appears to be a minor one, and that aging is "in large measure a purely chemical process".

The major bacterium identified on cured tobacco leaves was *Bacillus megatherium*; other microorganisms present were fungi like *Penicilla* and *Aspergilli* (Reid et al. 1944). During fermentation, the fungi disappeared, *Micrococcus candicans* and *Bacillus mesentericus–vulgatus* group increased. Reid et al. 1944 concluded that the fungi initially present on cured leaf were readily destroyed in good fermentation. It was concluded that the chemical conversions during aging were intensified (Tso 1979). It is likely

that enzymes, microorganisms, and catalysts all play a part in the process of flue-cured tobacco fermentation.

It was reported that in flue-cured tobacco leaves average populations of fungi per sample over 3 years ranged from 0 to 1,528,500 colonies per gram of tobacco (Welty 1972). The amount and the number of species of microorganisms on flue-cured tobacco leaves were found to be high in the early period of aging, but gradually decreased with aging (Zhao et al. 2000; Qiu et al. 2003–2004). The dominant microflora of bacteria, antinomyces and molds were *Bacillus*, *Streptomyces*, *Aspergillus* and *Penicillium*, though the amount of actinomyces and molds were less.

Those above studies employed traditional culture-dependent methods that might cause the lose of microflora multiformity because only a fraction (0.01% to 10%) of the bacteria known in natural habitats can be cultivated on laboratory media (Atlas and Bartha 1987). So the dominant microorganisms determined by traditional methods may not be the real ones.

In recent years, new technologies developed in molecular biology have overcome the culturing process and not only can analyze the cultured microorganisms but also can analyze the uncultured ones. Those methods employ PCR amplification of microbial 16S or 18S rDNA and then analyze the microflora multiformity by denaturing gradient gel electrophoresis (DGGE) technology. At present, PCR–DGGE was widely used on samples from soil, water, rhizosphere, and leaves surface (Ellis et al. 2003; Hoefel et al. 2005; Duineveld et al. 2001; Yang et al. 2001). Yang et al. (2001) working with leaves' surface microorganisms of corn, cotton, and green bean concluded that the microbial phyllosphere communities on the leaves' surface are more complex than previously thought based on conventional culture-based methods.

There are few reports about the microflora multiformity on aging tobacco leaves by PCR–DGGE technology. In this study, we explored the bacteria multiformity on aging flue-cured tobacco leaves by PCR–DGGE technology, and sequenced the 16S rDNA V3 regions of the dominant bacteria.

Materials and methods

Leaf sampling

Leaf samples of aging flue-cured tobacco variety Zhongyan 100, NC89, and Zhongyan 101, were collected from Biyang Tobacco Co. (Biyang County, Henan Province, China) and Xiangcheng Tobacco Co. (Xiangcheng County, Henan Province, China), respectively. All samples were of grade C3F. Leaf samples were placed at room temperature ranging from 0°C to 30°C to age, and were selected for sampling after 0, 3, 6, 9, and 12 months, respectively.

Extraction and purification of DNA from phyllosphere microorganisms on flue-cured tobacco leaves

Thirty grams leaves were divided into six equal parts and placed in six conical flasks with 300 ml sterilized water and shaken for 1 h, 210 rpm. The mixture was then filtered through two layers of pledget and bacteria were collected from the filtrate by centrifugation at 30,000×*g* for 15 min. Total DNA from the phyllosphere microorganisms was isolated following Lin and Feng (2004) and purified with Vitagen Kit following the manufacturer's description.

Bacterial 16S rDNA PCR amplification

A 230-bp DNA fragment in the V3 region of the small subunit ribosomal RNA gene from the purified genomic DNA of the bacterial communities from tobacco leaves of different aging periods were amplified by using primers set F357 and R518 as described by Muyzer et al. (1993). Amplification was performed with a PTC-200 Thermocycler (MJ Research Inc., Watertown, MA) in 0.2 ml tube using 50 μL reaction volume. The reaction mixture contained 5 μL of 10× reaction buffers, 2 mM of $MgCl_2$, 200 μM of dNTPs, 0.4 μM of each primer, 2.5 μL of template (approximately 50 ng), 2.5 U Taq DNA polymerase (Promega), and 3,000 ng of bovine serum albumin. Double distilled water was added to have a final volume of 50 μL. The cycling program was as follows: a 94°C of initial denaturing step for 5 min, and 21 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min with a touchdown of 0.5°C every second cycle until the annealing temperature of 56°C. The next 10 cycles was 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and the final step was 7 min extension step at 72°C. Amplification products were analyzed first by electrophoresis in 1.5% (w/v) agarose gels and by ethidium bromide staining.

DGGE analysis

DGGE was performed with a Dcode Universal Mutation Detection System (Bio Rad). 30 μL of the PCR products from phyllosphere samples was loaded onto a 10% (w/v) acrylamide gel (acrylamide/bis solution, 37.5:1; sigma) containing a linear chemical gradient ranging from 35% to 55% denaturant [7 M urea and 40% (v/v) formamide]. Electrophoresis was performed at a constant voltage of 120 V and a temperature of 62°C in a DGGE chamber containing approximately 7 l of 1×TAE buffer (0.04 M Tris base, 0.02 M sodium acetate, and 10 mM EDTA; pH adjusted to 7.4). After electrophoresis, the gels were stained for 40 min with SYBR Green I DNA stain solution (1:10,000 dilution; Molecular Probes) and photographed using a UV transilluminator (Vilber Lourmant Ltd., France).

Recovery of bands from DGGE gels and sequence analysis

DGGE bands, which were brighter and had higher frequency of appearance in each sample, were selected for excision and nucleotide sequence determination. For each band selected, only the middle portion was excised with a sterile razor, and slices (approximately 30 mg in wet weight) were placed in 1.5 ml polypropylene tubes. After washing the slices two times with 70% cold ethanol, 30 μL double distilled water was added to each tube. The tubes were then frozen and incubated for 30 min at 55°C to diffuse DNA. After centrifugation at 5,000×*g* for 1 min, 3 μL of diffused DNA solution was used as the template for re-amplification performed under the conditions described above. 3 μl of each reaction mixture was again subjected to DGGE analysis to confirm the product purity and melting behavior of the band recovered. Some DNA samples still contained mixed products because multiple DGGE bands emerged again in DGGE gels. In this condition, it was necessary to perform a second round of excision to be assured that bands of the same mobility were actually identical in sequence.

To analyze the sequence of selected bands, a 2.5 μL of diffused DNA supernatant was used as template DNA for a subsequent PCR amplification with primers R518/F357. PCR reaction conditions were as follows: after initial denaturation at 95°C for 4 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min were performed, and then the reaction mixture was kept at 72°C for 5 min. As described above, a portion of the PCR product was subjected to agarose gel electrophoresis to confirm product recovery and to estimate product length. After purification, the PCR products were cloned into *E.coli* JM109 using the pMD18-T vector system as described by the manufacturer (Takara Ltd, Dianlian, China). Clones with the correct size inserts were sequenced. These short fragments were compared to sequences in the NCBI nucleotide database, and the closest match of known phylogenetic affiliation was used to assign the bands to taxonomic groups.

Cluster analysis

Cluster analysis was performed on the band profiles from DGGE gels to explore the similarities in bacteria community structure of aging flue-cured tobacco leaves, as described by Yang et al. (2001).

Results

Analysis on bacterial population shift by DGGE

By using DGGE analysis of microbes on flue-cured tobacco leaves in different aging periods, distinct 16S rDNA banding patterns were observed (Fig. 1). Every tobacco leaf samples of the three varieties had different band profiles in different aging periods, respectively, but there were five dominant bands A, B, C, D, and E in all tobacco leaves' samples of the three varieties. For variety Zhongyan 100, there were basically no changes in band number and intensity in the early aging periods from 0 to 6 months, which showed that bacterial communities remained stable. But after 9 months aging, there were significant changes in the band profiles. Some bands disappeared and other bands became weak, while no new bands appeared. For example, the intensity of As and Ds did not have any obvious change during 0 to 12 months aging. The intensity of B reached a peak in the ninth month of aging while those of Cs and Es in 9 to 12 months aging were higher compared to those in 0 to 6 months.

The changes of the band profiles of NC89 and Zhongyan 101 were similar to Zhongyan 100 during all aging periods except the intensity of band As of NC89 and Zhongyan 101, band Cs of NC89 and Zhongyan 101, and band Ds of Zhongyan 101; The intensity of band As of NC89 and Zhongyan 101 in 9 to 12 months aging were higher than those in 0 to 6 months. No change was observed for the intensity of band Cs for NC89 during all aging periods while it reached bottom in the sixth month of aging for Zhongyan 101. The intensity of band Ds of Zhongyan 101 increased in 9 to 12 months aging compared to 0 to 6 months aging. The increase in the intensity of a band meant the numbers of the bacteria of a species or a group of species increased.

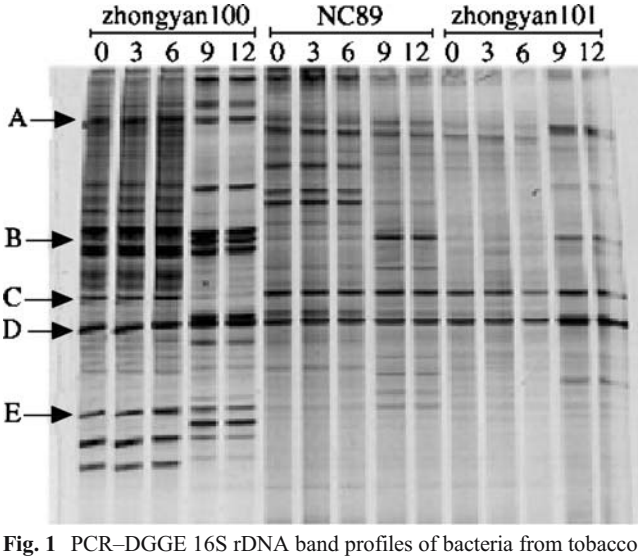

Fig. 1 PCR–DGGE 16S rDNA band profiles of bacteria from tobacco leaves of the three varieties Zhongyan 100, NC89, and Zhongyan 101. 0, 0 month aging; 3, 3 months aging; 6, 6 months aging; 9, 9 months aging; 12, 12 months aging

Structure of microbial communities on the aging tobacco leaves

The band profiles in Fig. 1 were analyzed by clustering methods to examine the relative similarities of bacterial communities on the aging tobacco leaves. Similar community structures were observed between 0 and 6 months aging, and between 9 to 12 months aging of tobacco leaves in every variety (Fig. 2), while low similarity were observed between 0 and 6, and between 9 and 12 months aging of every variety. It is likely that after 6 months aging there were distinct changes in the species and numbers of bacteria on flue-cured tobacco leaves. There were a certain similarities in bacterial communities (similarity to 63%) in 0 to 6 months aging among the three varieties.

Identification of dominant bacterial species

Five dominant 16S rDNA DGGE bands, A, B, C, D and E, were isolated, cloned, and sequenced (Table 1). Band A had a DNA sequence with 93% similarity rate to that of GenBank accession no. AY294222, which was most similar to the 16S rDNA V3 region of described microbial species, *Bacteriovorax* sp. EPC3; and B had a DNA sequence with similarity rates up to 98% to that of GenBank accession no. AJ880767, which was most similar to the 16S rDNA V3 region of described microbial species *Bacillus megaterium*; and C, D, and E had DNA sequence with similarity rate of 100%, 99%, and 100% to that of GenBank accession nos. AY758563, AY939036, and AY095384, which were all uncultured bacteria.

Discussion

There were about eight genera bacteria on aging flue-cured tobacco leaves reported based on culture-dependent methods (Zhao et al. 2000; Zhu et al. 2001). This was perhaps a simple profile compared with the results analyzed using DGGE of PCR-amplified 16S rDNA fragments in our study. There were 18–25 DGGE bands of every tobacco aging leaves in the three varieties (Fig. 1). We found five

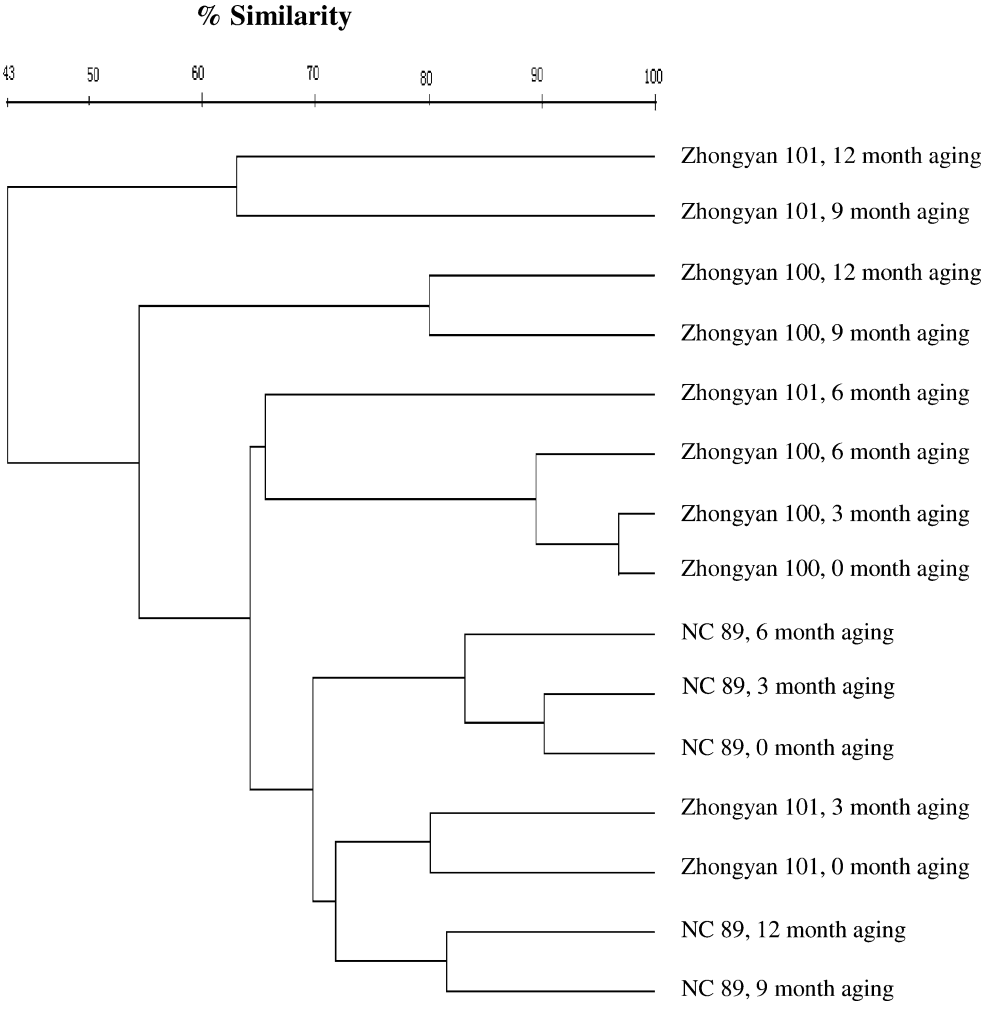

Fig. 2 Cluster analysis of 16S rDNA band profiles for bacteria from the aging flue-cured tobacco leaves

Table 1 Alignment of sequenced clone to its most similar GenBank sequences

Sequenced clones	Length of 16S rDNA V3 fragments (bp)	GenBank accession no.	Aligned site in GenBank sequence	Similarity rate (%)	Source of the most similar GenBank sequence
A	195	AY294222	301–495	93	*Bacteriovorax* sp. EPC3
B	194	AJ880767	345–538	98	*Bacillus megaterium*
C	169	AY758563	1–169	100	Uncultured *Sphingomonas* sp
D	194	AY939036	279–447	99	Uncultured bacterium
E	174	AY095384	299–472	100	Uncultured yard-trimming-compost bacterium clone S-12

genera bacteria when the five dominant bands were sequenced, among them four genera had not been described in the previous culture-based studies.

The number and the intensity of DGGE bands were higher in tobacco leaves of 0 to 6 months aging than 9 to 12 months aging (Fig. 1). That was probably responsible for the enzymes profiles in aging flue-cured tobacco leaves. The activities and isoenzymes belts of polyphenol oxidase, peroxidase, amylase and protease in flue-cured tobacco leaves gradually increased during 0 to 6 months aging, then decreased gradually during 9 to 12 months aging (Zhao et al. 2006).

Not only were there many microorganisms but also the microorganisms could grow on aging flue-cured tobacco leaves despite that the moisture content of aging flue-cured tobacco leaves decreased to 11–13% (Zhao et al. 2000). For applying bio-augmentation technique to shorten flue-cured tobacco aging period and improve tobacco leaves quality, scientists have been surveying the dominant group of species by culture-dependent methods (Qiu et al. 2000; Zhu et al. 2001).

Since most of the bacteria occurring in natural habitats could not be cultivated, it probably leads to non-accurate results using culture-dependent methods to reveal the dominant groups of species on aging flue-cured tobacco leaves. In this study, we discovered that three out of five dominant species on aging flue-cured tobacco leaves were uncultivated bacteria. It needs further study to determine whether cultivated or uncultivated dominant species should be focused in the study of flue-cured tobacco leaf aging process, and how to use uncultivated dominant species. However, DGGE analysis tends to reveal only the dominant microflora, which is a serious deficiency if minor organisms may make important biological contributions to an ecosystem as other studies indicated (Yang et al. 2001). So, there is still the need to explore new and more efficient methods in using any microorganisms to augment flue-cured tobacco leaves aging process. Furthermore, it is good that there were certain similarities of bacterial communities (similarity up to 63%) in tobacco leaves during 0 to 6 months aging among the three varieties, which means those similar bacterial species probably make important contributions to flue-cured tobacco aging process.

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