CHANGES IN THE COMPOSITION OF THE BACTERIAL FLORA ON TRAY-PACKAGED PORK DURING CHILLED STORAGE

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***Abstract*—In this study, a PCR-denaturing gradient gel electrophoresis (DGGE) was used to investigate the changes in the composition of the bacterial population of tray-packaged pork during the chilled storage. DGGE analysis of the V3 and V6-V8 regions of the 16S rRNA gene showed that *Pseudomonas* were the predominant bacteria at the end of the monitoring. The DGGE results of 16S rDNA-V3 region also indicated that *Brochothrix thermosphacta* became one of the dominant component of the spoilage microflora in the end. The results of PCR-DGGE obtained from different variable regions of the 16S rRNA gene were not completely the same.**

***Index Terms*—Bacterial community composition; Chilled storage; PCR-DGGE; Tray-packaged pork.**

I. INTRODUCTION

Meat is readily contaminated and is an ideal substrate for various spoilage and pathogenic microorganisms due to its high water content and abundance of essential nutrients (Jackson, Acuff, & Dickson, 1997; Jay, 2000). The composition of the bacterial flora of meat and the development of the spoilage flora under different storage conditions have been traditionally studied on the basis of cultivation, isolation and identification by phenotypic means (Blickstad & Molin, 1983; Blixt & Borch, 2002; Dainty, Shaw, & Roberts., 1983; Dainty & Mackey, 1992). Recently, an alternative molecular method, denaturing-gradient gel electrophoresis (DGGE) which is independent of cultivation, has been successfully applied to investigate the microbiology of fermented foods (Ampe, Omar, Moizan, Wacher, & Guyot, 1999; Cocolin, Manzano, Cantoni, & Comi, 2001) and fresh beef meat (Ercolini, Russo, Torrieri, Masi, & Villani, 2006; Fontana, Cocconcelli, & Vignolo, 2006; Russo, Ercolini, Mauriello, & Villani, 2006). The aim of this study was to use the PCR-DGGE to investigate the changes in the composition of the bacterial population of tray-packaged pork during the chilled storage.

II. MATERIALS AND METHODS

Sampling

Three pork loin cuts, labeled as group A, group B and group C, were removed from pork carcasses at 24 h post-mortem in a commercial meat plant. Each refrigerated meat cut was divided into five small pieces and each piece was immediately placed on one Styrofoam tray, which was overwrapped with an oxygen permeable polyethylene film (Hongbaoli Package Company, China; O_2 transmission rate, 8000 cm^3 / m^2 / atm in 24h at 23℃). The tray-packaged pieces of pork were stored at 4℃. One pack from each group at 0, 3, 6, 9 and 12 days of storage was selected for analysis, respectively.

DNA Extraction

Total DNA was extracted from pork samples. From each sampling point, 10 g pork samples of each pack were homogenized in a stomacher tube with 100 ml of saline peptone water, followed by agitation for 30 min at room temperature. The tubes were centrifuged for 5 min at 200 × g and the supernatant (30 ml) was transferred to a 50 ml sterile centrifuge tube, and a further centrifugation was carried out at 12,000 × g for 10 min. The pellet was resuspended in 150 µl of Buffer S of Axyprep Bacterial Genomic DNA Miniprep kit (Axygen, USA) and transferred to a sterile 2 ml tube. Twenty microlitres of lysozyme (50mg/ml) (Sigma) was added and incubated for 1h at 37℃. Thirty microlitres of proteinase K (10mg/ml) (Tiangen, China) was added and incubated for 2.5h at 55℃. The preparations were subjected to DNA extraction using Axyprep Bacterial Genomic DNA Miniprep kit (Axygen, USA) according to the manufacturer's instructions.

Nested PCR Amplification

All primers used in this study are listed in Table 1. The PCR amplification was performed by using Mastercycler ep (Eppendorf, Germany) according to the manufacturer's instructions. Nested PCR was used for 16S rDNA-V3 amplification of total DNA. In the first round of PCR, the primers 27f and 798r (Table 1) were designed to amplify the approximate 800 bp 16S rRNA gene fragments (Lane, 1991; Li, He, Wu, & Jiang, 2006). The amplification reactions were carried out in a 25 µl reaction volume containing GoTaq Green Master Mix (2×) 12.5 µl, each primer 1 µl (0.4

μM), DNA template 1μl, and ddH_2O 9.5 μl. The following PCR program was used: 95℃ for 2min, and 25 cycles of 94℃ for 1 min, 58℃ for 1 min, 72℃ for 2 min and 72℃ for a 2 min final extension. PCR products were further purified by the Wizard SV Gel and PCR Clean-up System (Promega, USA) according to the manufacturer's protocol. Subsequently, a second PCR was performed, using the purified amplicons of the first PCR as template DNA. The second round of PCR was carried out by using primers 338f-GC and 518r spanning the V3 region of the 16S rRNA gene (Muyzer, De Wall, & Uitterlinden, 1993; Ampe et al., 1999). The amplification reactions were conducted in a 50 μl reaction volume containing GoTaq Green Master Mix (2×) 25 μl, each primer 1 μl (0.2 μM), DNA template 1μl, and ddH_2O 22 μl. To increase the specificity of the amplification and reduce the formation of spurious byproducts, a "touchdown" PCR was carried out. The initial annealing temperature was 65℃,which was 10℃ higher than expected and then decreased by 0.5℃ every second cycle until the touchdown temperature of 55℃ was reached. 15 additional cycles were then carried out at 55℃. A denaturation step of 94℃ for 1 min was used and extension was carried out at 72℃ for 3 min with a final extension at 72℃for 10 min.

Nested PCR was used for 16S rDNA-V6-V8 amplification of total DNA. In the first round of PCR, the primers 27f and 1492r (Table 1) were designed to amplify the approximate 1500 bp 16S rRNA gene fragments (Wilson, Blitchington, & Greene, 1990; Lane, 1991). The amplification reactions were carried out in a 25 μl reaction volume containing GoTaq Green Master Mix (2×) 12.5 μl, each primer 0.5 μl (0.2 μM), DNA template 1μl, and ddH_2O 10.5 μl. Cycling conditions were: 95℃ for 3 min, and 30 cycles of 95℃ for 30 s, 53℃ for 30 s, 72℃ for 1 min and 72℃ for a 7min final extension (Wilson et al., 1990). A second PCR was performed, using the purified amplicons of the first PCR as template DNA. Primers 968f-GC and 1401r were used to amplify the V6-V8 regions of the 16S rRNA gene (Nübel et al., 1996). The amplification reactions were carried out in a 50 μl reaction volume containing GoTaq Green Master Mix (2×) 25 μl, each primer 1 μl (0.2 μM), DNA template 1μl, and ddH_2O 22 μl. The following PCR program was used: 94 ℃ for 5 min, and 35 cycles of 94℃ for 30 s, 56℃ for 20 s, 68℃ for 40 s and 68℃ for a 7 min final extension.

Aliquots of 5 μl were analyzed by electrophoresis on a 1.2% agarose gel (w/v) containing ethidium bromide to determine the size of the PCR products.

DGGE Analysis

PCR amplicons were separated by DGGE according to the specifications of Muyzer et al. (1993) by using the Dcode system (Bio-Rad, Hercules, CA) with the following modifications. Polyacrylamide gels (dimensions, 160 × 160 × 1 mm) consisted of 8% (wt / vol) polyacrylamide gels (37.5:1 acrylamide / bisacrylamide). Denaturing acrylamide of 100% was defined as 7 M urea and 40% formamide. Denaturing ranges of 35-55% and 38-51% were used for the separation of the PCR amplicons of the V3 region and V6-8 regions, respectively. A 100% denaturant acrylamide was defined as 7M urea and 40% formamide. Electrophoresis was performed for 10 min at 200 V and then for 16 h at 85 V in a 0.5× TAE buffer at a constant temperature of 60℃. Gels were stained with ethidium bromide (0.5 mg/l) for 10 min, then rinsed for 20 min in milli-Q water and photographed with UV transillumination using the GelDoc 2000 system (Bio-Rad).

Cloning and Sequencing of PCR-amplified Products

Full-length bacterial 16S rRNA genes from pork samples of 0-day and 12-day in group C were amplified by PCR with primers 8f and 1510r (Lane, 1991). PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega, USA) and cloned in *Escherichia coli* JM109 by using the pGEM-T vector system (Invitrogen). After overnight growth, single colonies of ampicillin-resistant transformants were picked, transferred to Luria broth medium and incubated at 37℃ overnight. One hundred microlitres of the above cultures were centrifuged and suspended in 100-μl Tris-EDTA buffer. Suspensions were boiled for 5min to lyse cells, and were then used as templates to perform PCR amplification using pGEM-T-specific primers T7 and Sp6 (Promega) to check the size of the cloned inserts. The plasmids containing inserts of *c.* 1.6 kb were used to amplify the V3 and V6-V8 regions of the 16S rRNA gene with the primer sets containing GC-clamp, respectively, as described above. The amplicons were then compared with bands derived from the same original samples by DGGE profiling. Amplicons corresponding to distinct bands of DGGE profiles were selected and then sequenced commercially (Invitrogen). Similarity searches of the GenBank DNA database were performed with the BLAST Search tool (Altschul et al., 1990).

Nucleotide sequence accession numbers

Sequences of pork bacterial 16S rRNA gene clones have been deposited in the GenBank database under accession numbers GQ422759 through GQ422769.

Table 1 Primers used in this study

Primer	Primer sequence (5′ to 3′)	Targeted regions in 16S rRNA gene	Reference
27f	AGA GTT TGA TCA/C TGG CTC AG		Lane, 1991
798r	CCA GGG TAT CTA ATC CTG TT		Li et al., 2006
1492r	ACG GYT ACC TTG TTA CGA CTT		Wilson et al., 1990
338f (GC^1)*	ACT CCT ACG GGA GGC AGC AG	V3 region	Ampe et al., 1999
518r	ATT ACC GCG GCT GCT GG		Muyzer et al., 1993
968f (GC^2)*	AAC GCG AAG AAC CTT AC	V6-V8 region	Nübel et al., 1996
1401r	CGGTGTGTACAAGACCC		Nübel et al., 1996
8f	CACGGATCCAGAGTTTGAT(C/T)(A/C)TGGCTCAG	Complete 16S rRNA gene	Lane, 1991
1510r	GTGAAGCTTACGGCTACCTTGTTACGACTT		Lane, 1991
T7	TAATACGACTCACTATAGG		Promega
Sp6	GATTTAGGTGACACTATAG		Promega

* A GC clamp was attached to the 5′ end of the primer. (GC^1) CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G; (GC^2) CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G.

III. RESULTS AND DISCUSSION

The microflora of aerobically stored meats has been extensively studied by means of the traditional cultivation methods. The initial, mainly mesophilic flora can vary due to sources of contamination (Dainty et al., 1983). *Micrococcus*, *Staphylococcus*, *Kurthia*, Enterobacteriaceae, *Moraxella* / *Acinetobacter*, *Flavobacterium*, coryneforms, *Pseudomonas*, *Lactobacillus* and *Brochothrix thermosphacta* (Dainty et al., 1983) are likely to be present. In this study, by means of DGGE analysis of the V3 region of the 16S rRNA gene, a biodiversity was found in the initial stages of the meat storage, but only *Achromobacter xylosoxidans* (band 8), *Ps. fluorescens* (band 9) and *Serratia marcescens* (band 10) (Table 2) were dominant bacteria. Meanwhile, DGGE analysis of the V6-V8 regions of the 16S rRNA gene also found that *Ps. fluorescens* (band B, Fig. 1b) was the dominant bacterium in fresh pork. *Staphylococcus* has been reported to occur on fresh meat but at lower levels (Blickstad and Molin, 1983) and it was also observed in the initial stage in the present study (band A, Fig. 1b).

The initial flora present in fresh meat has been shown to change during chilled-storage under aerobic conditions to psychrophilic Gram-negative rods, such as *Pseudomonas*, *Acinetobacter* and *Psychrobacter* (Dainty and Mackey, 1992). In this study, the DGGE results of different variable regions of 16S rRNA gene all indicated that *Pseudomonas* became the dominant bacterium at the end of the monitoring (Fig. 1), which was in agreement with results obtained in previous studies by using traditional cultivation methods (Dainty et al, 1983; Labadie, 1999). However, the identification of *Pseudomonas* at the species level could not be achieved in this study due to the fact that it is impossible to obtain unequivocal identifications for most of the DGGE fragments. Thus, the V3 and V6-V8 regions of the 16S rRNA gene used in this study may not be distinguished among the species of *Pseudomonas*, suggesting the limitation in the use of a 16S-based PCR-DGGE approach for identification of *Pseudomonas* in the meat ecosystem ((Ercolini et al, 2006). The psychrotrophic species *B. thermosphacta* represents a significant component of the spoilage microflora of meat stored aerobically (Labadie, 1999) and it became another dominant bacterium at the end of the monitoring in the present study (Fig. 1a). The differences between the DGGE results of the V3 region and the V6-V8 regions maybe due to the bias in template-to-product ratios in multitemplate PCR (Polz & Cavanaugh, 1998).

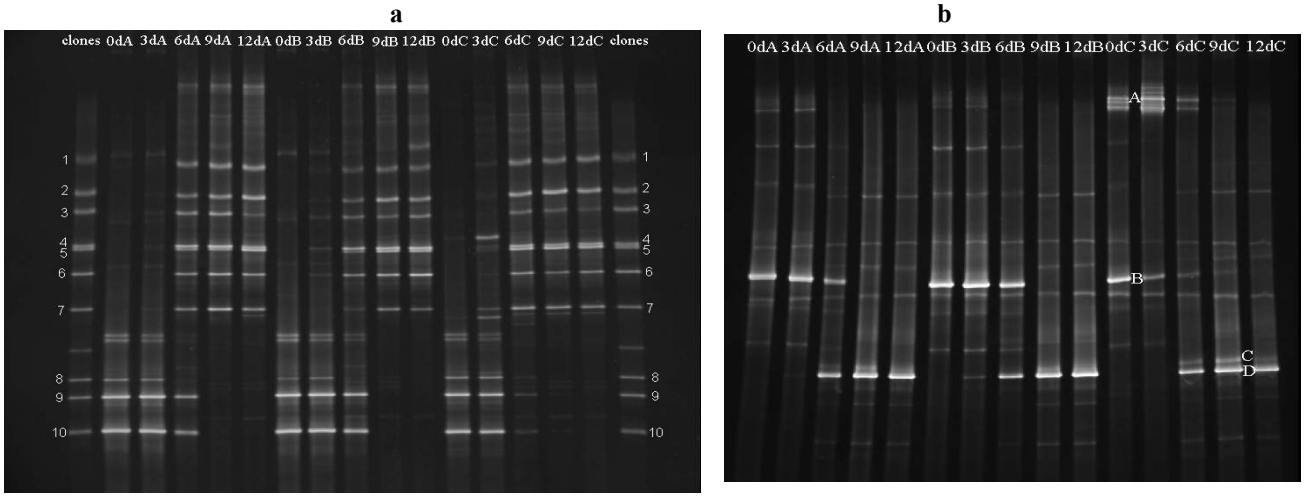

Fig. 1. DGGE profiles of PCR amplicons of the V3 region (a) and V6-V8regions (b) of the 16S rRNA gene of the DNA extracted directly from pork.

Table 2 Closest matches and percentage similarity of GenBank database sequences to sequences of clones associated with bands in DGGE profiles of meat samples.

Bands No.*	Clone	Closest relative	Length (bp)	Similarity %	Accession No.
1	A3	*Pseudomonas* sp.	1500	100	GQ422759
2	A4	*Pseudomonas* sp.	1500	99.9	GQ422760
3	A8	*Pseudomonas* sp.	1500	99.7	GQ422761
4	A30	*Pseudomonas fluorescens*	1500	99.9	GQ422762
5	A1	*Pseudomonas* sp.	1500	99.7	GQ422763
6	A16	*Pseudomonas* sp.	1500	99.5	GQ422764
7	A7	*Brochothrix thermosphacta*	1511	100	GQ422765
8	B24	*Achromobacter xylosoxidans* / *Achromobacter* sp.	1494	100	GQ422766
9	B25	*Pseudomonas fluorescens*	1505	99.8	GQ422767
10	B33	*Serratia marcescens*	1505	100	GQ422768
A	B37	*Staphylococcus kloosii*	1515	99.7	GQ422769
B	B25	*Pseudomonas fluorescens*	1505	99.8	GQ422767
C	A7	*Brochothrix thermosphacta*	1511	100	GQ422765
D	A1	*Pseudomonas* sp.	1500	99.7	GQ422763

* Band numbers as indicated in Fig 1.

IV. CONCLUSION

In this study, PCR-DGGE was used to investigate the changes in the composition of the bacterial population of tray-packaged pork during chilled storage. The initial flora present in the fresh meat was complex, with *Pseudomonas* and *Brochothrix thermosphacta* becoming the predominant bacteria at the end of the monitoring. In conclusion, the PCR–DGGE technique as a culture independent method is applicable to monitoring bacterial population dynamics in tray-packaged pork. However, it should be considered that the results of PCR-DGGE obtained from different variable regions of the 16S rRNA gene may be not completely the same.

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