AMPLIFIED FRAGMENT LENGTH POLYMORPHISM ANALYSIS IN STRAIN TYPING AND IDENTIFICATION OF *LISTERIA* AND *CLOSTRIDIUM* SPECIES

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Riikka Keto-Timonen

ACADEMIC DISSERTATION

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TO MY FAMILY

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ABBREVIATIONS

AFLP	amplified fragment length polymorphism
ARDRA	amplified ribosomal DNA restriction analysis
ATCC	American Type Culture Collection
BHI	brain heart infusion
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
D	discrimination index
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
fAFLP	fluorescent amplified fragment length polymorphism
ITS	internal transcribed spacer
MLEE	multilocus enzyme electrophoresis
MLSSCP	multilocus single-strand conformation polymorphism
MLST	multilocus sequence typing
MLVA	multiple-locus variable-number tandem repeat analysis
MRP	macro-restriction pattern
PCR	polymerase chain reaction
PCR-REA	polymerase chain reaction and restriction enzyme analysis
PFGE	pulsed-field gel electrophoresis
PIV	Tris-NaCl buffer
RAPD	randomly amplified polymorphic DNA
rep-PCR	repetitive sequence-based PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
sAFLP	single-enzyme AFLP
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
*slp*AST	surface layer protein A gene sequence typing
TE	Tris-HCl, EDTA buffer
TE-AFLP	three-endonuclease amplified fragment length polymorphism
VNTR	variable number tandem repeat

ABSTRACT

Fluorescent amplified fragment length polymorphism (fAFLP) analysis was tailored for optimal characterization of *Listeria monocytogenes* and *Clostridium botulinum*. Of the tested combinations, the enzyme coupling *Hin*dIII – *Hpy*CH4IV with primer combinations Hind-A and Hpy-A, and Hind-C and Hpy-A for *L. monocytogenes* and *C. botulinum*, respectively, showed evenly distributed banding patterns in the optimal size range and detected polymorphism between closely related strains and were thus selected for further analysis.

The suitability of AFLP analysis to type *L. monocytogenes*, *C. botulinum* and *Clostridium perfringens* at strain level was evaluated. AFLP proved to be a highly reproducible, easy-to-use, relatively fast and highly discriminative approach. In addition, all strains were typeable by AFLP, and thus, the method seemed to overcome the problem of extracellular DNase production detected in some clostridial strains. The discriminatory power of AFLP was shown to equal that of pulsed-field gel electrophoresis (PFGE) for *L. monocytogenes*. By combining the results of AFLP and PFGE, the subtype discrimination was further improved. AFLP was shown to be a suitable tool also for *C. botulinum* group identification.

Since phenotypic identification of *Clostridium* isolates is laborious, the suitability of AFLP for genomic species identification was assessed. The AFLP technique was applied to 129 strains representing 24 different *Clostridium* species. AFLP differentiated all species tested, except for *Clostridium ramosum* and *Clostridium limosum*, which clustered together at the 45% similarity level. *C. botulinum* strains showed wide genetic diversity and were divided into seven species-specific clusters, while other species were divided into single species-specific clusters or occupied separate positions. AFLP also differentiated between *L. monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi* species. If AFLP profiles of well-defined strains are collected in identification libraries, the database can be a valuable additional tool for identification of *Clostridium* and *Listeria* species. Due to high throughput of samples, AFLP proved to be especially suitable for screening large numbers of isolates.

Contamination routes of *L. monocytogenes* were traced in a chilled food processing plant producing ready-to-eat and ready-to-reheat meals during an 8-year period by AFLP. Clearly different contamination statuses were observed in the three compartments (I-III) of the plant. Compartment I, which produced cooked meals, was heavily contaminated with three persistent AFLP types, whereas compartment II, which produced uncooked chilled food, was contaminated with both persistent and sporadic AFLP types. The equipment of compartment III was free of contamination. Cleaning routines, product type and degree of compartmentalization seemed to have

an influence on the contamination status in compartments that produced cooked meals. In addition, raw materials were shown to cause product contamination in compartment II. Thus, special attention should be paid to quality control of raw ingredients when uncooked ready-to-eat meals are produced. In compartment II, reconstruction of the production line was demonstrated to reduce prevalence rates of *L. monocytogenes* and to eliminate two persistent AFLP types.

L. monocytogenes strains causing persistent plant contamination and sporadic strains were analysed using AFLP and PFGE. Persistent strains showed 15 genotypes, 13 of which were specific for persistent strains, whereas sporadic strains were divided into 35 genotypes, 33 of which were only associated with sporadic strains. Although persistent strains differed from sporadic strains, no specific evolutionary lineage of persistent strains was observed.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by Roman numerals I-V:

I. Keto-Timonen, R., Autio, T., and Korkeala, H. 2003. An improved amplified fragment length polymorphism (AFLP) protocol for discrimination of *Listeria* isolates. System. Appl. Microbiol. 26: 236-244.

II. Keto-Timonen, R., Nevas, M., and Korkeala, H. 2005. Efficient DNA fingerprinting of *Clostridium botulinum* types A, B, E, and F by amplified fragment length polymorphism analysis. Appl. Environ. Microbiol. 71: 1148-1154.

III. Keto-Timonen, R., Heikinheimo, A., Eerola, E., and Korkeala, H. 2006. Identification of *Clostridium* species and DNA fingerprinting of *Clostridium perfringens* by amplified fragment length polymorphism analysis. J. Clin. Microbiol. 44: 4057-4065.

IV. Keto-Timonen, R., Tolvanen, R., Lundén, J., and Korkeala, H. 2007. An 8-year surveillance of the diversity and persistence of *Listeria monocytogenes* in a chilled food processing plant analyzed by amplified fragment length polymorphism. J. Food. Prot. 70: 1866-1873.

V. Autio, T., Keto-Timonen, R., Lundén, J., Björkroth, J., and Korkeala, H. 2003. Characterization of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). System. Appl. Microbiol. 26: 539-545.

1. INTRODUCTION

In epidemiological studies, techniques that effectively discriminate between individual bacterial strains are essential. In outbreak situations, strain typing is needed both to distinguish outbreak-associated cases from sporadic cases and to trace the vehicle of infection. Similarly, to be able to implement improved foodborne pathogen control strategies, the contaminating bacteria must be traced back to their source in the food processing plant. Methods based on the phenotypic characteristics of bacteria have traditionally been used for this purpose. The drawback of these methods is their restricted resolution. In addition, problems with typeability and reproducibility have been linked to many phenotyping techniques (Maslow *et al*. 1993, Olive and Bean 1999).

Genotyping offers several advantages compared with conventional phenotyping techniques. In theory, since all bacteria have DNA, they should be typeable by genotyping methods. Genomic DNA is very stable, thus being unaffected by environmental and cultural conditions, which may have an influence on the expressed phenotypic characteristics of bacteria. In general, the discriminatory power of DNA-based typing methods is also higher than that of phenotyping techniques (Farber 1996).

Numerous methods, including pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), ribotyping, polymerase chain reaction (PCR)-based typing methods, *e.g.* randomly amplified polymorphic DNA (RAPD), DNA microarray typing and sequence-based analysis, *e.g.* multilocus sequence typing (MLST), have been utilized in bacterial genotyping (Farber 1996, Power 1996, Borucki *et al*. 2003a, Meays *et al*. 2004, Torpdahl *et al*. 2005). However, large variation exists among the different genotyping methods in their discriminatory power, reproducibility and ease of standardization (Van Belkum *et al*. 2001, Meays *et al*. 2004). Combining high discriminatory power and reproducibility with ease of performance and speed is also problematic (Lindstedt *et al*. 2000c). Selecting the most suitable genotyping method for different kinds of investigations is therefore challenging. Recent developments in molecular techniques necessitate an ongoing need to tailor new genotyping methods for optimal characterization of different bacterial species and to evaluate their performance and suitability for research purposes.

Listeria monocytogenes and *Clostridium botulinum* are pathogens that can cause the rare but severe foodborne diseases of listeriosis and botulism, respectively (Hatheway 1995, Ramaswamy *et al*. 2007). On the other hand, enterotoxin-producing *Clostridium perfringens* type A is considered to be one of the most important causes

of bacterial-origin food poisoning (Brynestad and Granum 2002, Lukinmaa *et al*. 2002, Lynch *et al*. 2006). A better understanding of the epidemiology of *L. monocytogenes*, *C. botulinum* and *C. perfringens* is needed to improve control of these foodborne pathogens and to enable the production of safe food. For epidemiological investigations, efficient genotyping techniques are required.

Amplified fragment length polymorphism (AFLP) analysis is a PCR-based fingerprinting method that was originally developed for typing of plants. The AFLP technique involves three steps: 1) genomic DNA is cleaved with two restriction enzymes, 2) ligation of restriction site-specific adapters occurs and 3) a subset of fragments is amplified by PCR (Vos *et al*. 1995). The enzyme and primer combinations used in the analysis have an effect on the discriminatory power of AFLP, and thus, the AFLP protocol needs to be tailored for each bacterial species separately. The suitability of the AFLP technique for characterizing *C. botulinum*, *C. perfringens* and *L. monocytogenes* has not been sufficiently evaluated. Several studies have utilized AFLP to differentiate between bacterial species (Huys *et al*. 1996a, Janssen *et al*. 1996, Duim *et al*. 2001, On *et al*. 2003). However, studies surveying the potential of the AFLP approach for identification of *Clostridium* and *Listeria* species have not been conducted.

Foodborne listeriosis has been linked especially to ready-to-eat food products that are refrigeration-stored for longer periods. High incidence rates of *L. monocytogenes* in prepared meals (Nørrung *et al*. 1999, Uyttendaele *et al*. 1999) and the persistence of *L. monocytogenes* in chilled food factories that produce ready-to-eat meals have been reported (Holah *et al*. 2004). Ready meals are often reheated in microwave ovens, which may reheat the food unevenly. Hence, if the meal is contaminated with *L. monocytogenes*, the organism can survive in cold spots and pose a health risk for the consumer. However, despite the increased consumption of ready-to-eat convenience foods and foods requiring minimal preparation time (Gandhi and Chikindas 2007), little is known about the contamination routes of *L. monocytogenes* in plants producing ready-to-eat and ready-to-reheat meals. Thus, further research is needed to enable production of *L. monocytogenes*-free ready meals.

2. REVIEW OF THE LITERATURE

2.1 Amplified fragment length polymorphism analysis

Amplified fragment length polymorphism (AFLP) analysis was originally patented and described by Zabeau and Vos (1993) and Vos *et al*. (1995). The AFLP technology is covered by patents and patent applications owned by Keygene N.V. (Wageningen, Netherlands), and therefore, license from Keygene N.V. is needed if the AFLP technology is used for commercial purposes. The method was originally developed for the typing of crop plants, but it can be used for fingerprinting DNA of any origin, including DNA of animals, plants, nematodes, protozoa, fungi and bacteria (Janssen *et al*. 1996, Savelkoul *et al*. 1999, Blears *et al*. 2000, Borst *et al*. 2003, Ball *et al*. 2004, Bensch and Åkesson 2005, Mikkonen *et al*. 2005, Sharma *et al*. 2006, de Valk *et al*. 2007).

AFLP has many applications in the field of microbiology. It is nowadays widely used for strain typing and classification (Hookey *et al*. 1999, van der Zwet *et al*. 1999, 2000, Duim *et al*. 2000, Nair *et al*. 2000, Zhao *et al*. 2000, Gebreyes and Altier 2002, On *et al*. 2004, Fearnley *et al*. 2005, Ryu *et al*. 2005, Melles *et al*. 2007), and several studies have utilized AFLP in outbreak and epidemiological investigations (Speijer *et al*. 1999, Jonas *et al*. 2000, Georgnaras *et al*. 2001, Lan and Reeves 2002, McLauchlin *et al*. 2002, Ip *et al*. 2003, Motiwala *et al*. 2003, Ruiz *et al*. 2003, van der Zee *et al*. 2003, Jureen *et al*. 2004, Melles *et al*. 2004, Spence *et al*. 2004, Coque *et al*. 2005, Imataki *et al*. 2006, Johnsen *et al*. 2006c, Wong *et al*. 2006). AFLP can also be used to track sources, survival and spread of bacterial contamination at farm level, in slaughterhouses and in food processing plants (Georgnaras *et al*. 1999, Fonnesbech Vogel 2001, Johnsen *et al*. 2006a, 2006b, Wieland *et al*. 2006, Wulff *et al*. 2006, Johannessen *et al*. 2007). In addition, AFLP has been used to study microbial diversity in contaminated ecosystems (La Rosa *et al*. 2006).

2.2 Principles of the AFLP method

AFLP analysis consists of three steps: DNA is digested with restriction enzymes, ligation of restriction site-specific adapters occurs and a subset of fragments is amplified by PCR (Vos *et al*. 1995).

The total genomic DNA is first digested using two restriction enzymes; a rare-cutter and a frequent-cutter (Fig. 1). The rare-cutter and frequent-cutter typically

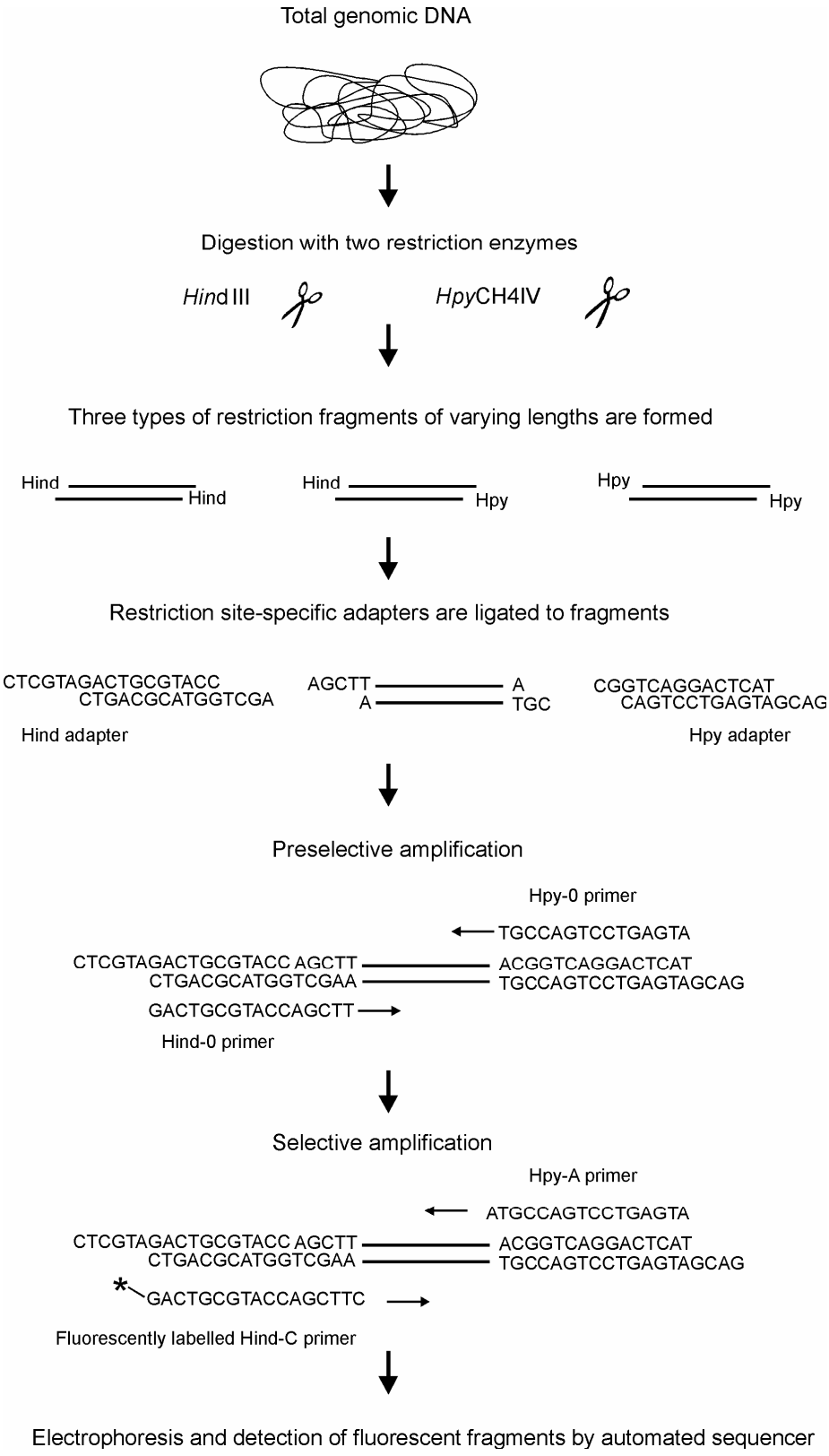

Figure 1. Schematic representation of fluorescent AFLP analysis using restriction enzymes *Hin*dIII and *Hpy*CH4IV and primers with one selective nucleotide during selective amplification.

have six- and four-nucleotide-long recognition sequences, respectively. A commonly applied restriction enzyme combination is *Eco*RI and *Mse*I, but several protocols using different enzyme combinations have been developed (Table 1). During digestion three kind of DNA fragments are formed (Fig. 1). The majority of the fragments are cut by the frequent-cutter at both ends, less fragments are cut by both the frequent-cutter and rare-cutter and a only a few fragments are cut by the rare-cutting restriction enzyme at both ends (Vos *et al*. 1995).

After digestion, restriction site-specific double-stranded nucleotide adapters (length 10-30 bp) are ligated to the ends of the DNA fragments (Fig. 1) (Vos *et al*. 1995, Blears *et al*. 1998). Adapters are complementary to the sticky end of the corresponding restriction site and designed so that the original restriction site is not restored after ligation. Therefore, once the adapter is ligated to the DNA fragment, digestion by the restriction enzyme is prevented. Formation of fragment-to-fragment products is also inhibited since restriction and ligation reactions occur simultaneously (Blears *et al*. 1998, Savelkoul *et al*. 1999).

Restriction fragments with specific adapters are amplified in two subsequent PCR reactions; preselective and selective PCR (Fig. 1) (Savelkoul *et al*. 1999). The preselective amplification provides an adequate amount of template DNA for selective amplification and reduces background smears in the AFLP patterns, especially when large genomes are analysed (Vos *et al*. 1995). The PCR amplifications are performed under highly stringent conditions to allow specific annealing of primers (Savelkoul *et al*. 1999). Typically, touch-down PCR is used during selective amplification (Vos *et al*. 1995). AFLP primers are complementary to the adapter and the restriction site sequence. In addition, 0-3 selective nucleotides are added to the 3'-end of the primer (Aarts *et al*. 1998, Blears *et al*. 1998). If nucleotides extending beyond the restriction site match the selective nucleotides of the primer, the restriction fragment is amplified. The selectivity of the primers is good when one or two selective nucleotides are used and acceptable when three selective nucleotides are included. However, addition of a fourth nucleotide results in reduced selectivity due to increased tolerance of mismatches during amplification (Vos *et al*. 1995).

A nearly linear correlation exists between the number of amplified fragments and the genome size, and thus, the size of the analysed genome affects the number of selective nucleotides used. In theory, addition of one selective nucleotide to the primer reduces the number of amplified fragments fourfold. Therefore, the complexity of the AFLP pattern can be reduced by addition of selective nucleotides. In general, the desired number of amplified fragments ranges from 50 to 100 (Vos *et al*. 1995). Typically, bacterial genome size is relatively small; known genome sizes of bacteria vary between 0.6 and 10 Mb (Moran 2002). When bacterial DNA is analysed by AFLP, the preselective amplification is often performed using primers without

selective nucleotides, and during selective amplification one selective nucleotide is added to both primers (Table 1).

During selective amplification the primer, which spans the rare-cutter restriction site, is labelled radioactively (Vos *et al*. 1995) or fluorescently (Desai *et al*. 1998, Koeleman *et al*. 1998). The labelled primer is totally consumed during PCR amplification, and thus, the amount of labelled primer rather than the number of PCR cycles serves as a limiting factor in the amplification process. Since an excess of PCR cycles is used, AFLP patterns of equal intensity are observed, although the template concentration may vary (Vos *et al*. 1995, van der Wurff *et al*. 1999). When the denatured fragments are electrophoresed on polyacrylamide gels, only the labelled fragments are visualized. Labelling also prevents the occurrence of double bands on the gels due to unequal mobility of the two strands of the amplified fragments (Vos *et al*. 1995). Alternative AFLP protocols utilizing silver staining (Geornaras *et al*. 1999, Briard *et al*. 2000, Wang *et al*. 2004) or chemiluminescent detection of fragments (Lin *et al*. 1999) have also been published.

At present, mainly fluorescent AFLP (fAFLP) is used (Table 1). In addition to the improved occupational safety, fAFLP is fast and easy to perform compared with radioactive AFLP (Koeleman *et al*. 1998, Coenye *et al*. 1999b). Furthermore, fAFLP enables analysis with an automated DNA sequencer, thus allowing accurate fragment sizing (±1 bp) if an internal size standard is included in every lane (Desai *et al*. 1998, Arnold *et al*. 1999b, Antonishyn *et al*. 2000). If different fluorescent labels are used, two samples can also be run simultaneously in the same lane (Antonishyn *et al*. 2000). AFLP analyses using both radioactively and fluorescently labelled primers have resulted in comparable clusters following numerical analysis of the profiles (Coenye *et al*. 1999b).

Table 1. AFLP protocols utilizing two restriction enzymes to type different bacterial species and comments on the discriminatory power of the techniques.

Species	Restriction enzyme combination	Primer combination in selective amplification [a]	F, R or S [b]	Comments on discriminatory power [c]	Reference
Acinetobacter baumannii	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	Discriminatory power of AFLP (D=0.94) equal to that of PFGE (D=0.94)	D'Agata *et al*. 2001
A. baumannii	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	NS	Spence *et al*. 2004
Acinetobacter spp.	*Hin*dIII – *Taq*I	Hin-A, Taq-AA	R	NS	Janssen *et al*. 1997
Acinetobacter spp.	*Eco*RI – *Mse*I	Eco-A, Mse-C	F, R	AFLP showed higher discriminatory power than ARDRA	Koeleman *et al*. 1998
Aeromonas spp.	*Apa*I – *Taq*I	Apa-A, Taq-A	R	NS	Huys *et al*. 1996a
Arcobacter butzleri	*Bgl*II – *Csp*6I	Bgl-0, Csp-A	F	NS	On *et al*. 2004
Bacillus anthracis	*Eco*RI – *Mse*I	16 different primer combinations used	R	NS	Keim *et al*. 1997
B. anthracis	*Eco*RI – *Mse*I	Eco-C, Mse-G	F	NS	Jackson *et al*. 1999
B. anthracis	*Eco*RI – *Mse*I	Eco-0, Mse-C	F	NS	Ryu *et al*. 2005
B. anthracis, Bacillus cereus, Bacillus thuringiensis	*Eco*RI – *Mse*I	Eco-C, Mse-G	F	NS	Radnedge *et al*. 2003, Hill *et al*. 2004
B. cereus	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	NS	Van der Zwet *et al*. 2000
B. thuringiensis	*Eco*RI – *Mse*I	Eco-AA, Mse-CAA	R	NS	Pattanayak *et al*. 2000
Bordetella spp.	*Apa*I – *Spe*I	Apa-0, Spe-0	F	AFLP showed higher discriminatory power than RAPD	Gzyl *et al*. 2005
Brucella spp.	*Eco*RI – *Mse*I	Eco-0, Mse-TC	F	NS	Whatmore *et al*. 2005
Burkholderia spp.	*Apa*I – *Taq*I	Apa-G, Taq-G	F, R	NS	Coenye *et al*. 1999b
Campylobacter coli	*Mfe*I – *Bsp*DI	Mfe-0 , Bsp-0	F	NS	Siemer *et al*. 2005
Campylobacter fetus	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	NS	Wagenaar *et al*. 2001

Table 1. Continued.

Species	Restriction enzyme combination	Primer combination in selective amplification [a]	F, R or S [b]	Comments on discriminatory power [c]	Reference
C. fetus	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	AFLP showed lower discriminatory power than MLST	Van Bergen *et al.* 2005a
C. fetus	*Mbo*I – *Dde*I	22 different primer combinations used	R	NS	Van Bergen *et al.* 2005b
C. jejuni	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	NS	Duim *et al.* 2000
C. jejuni	*Bgl*II – *Mfe*I	Blg-0, Mfe-0	F	AFLP showed better discriminatory power than PFGE and PCR-RFLP	Lindstedt *et al.* 2000c
C. jejuni	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	AFLP and PFGE were equally discriminative	Hänninen *et al.* 2001
C. jejuni	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	Similar grouping of strains obtained by AFLP and MLST	Schouls *et al.* 2003
C. jejuni	*Mbo*I – *Dde*I	64 different primer combinations used	R	NS	Godschalk *et al.* 2006
C. jejuni and *C. coli*	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	NS	Duim *et al.* 1999
C. jejuni and *C. coli*	*Bgl*II – *Csp*6I	Bgl-0, Csp-A	F	NS	Kokotovic and On 1999
C. jejuni and *C. coli*	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	AFLP showed higher discriminatory power than PFGE, flagellin typing and ribotyping	De Boer *et al.* 2000
C. jejuni and *C. coli*	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	AFLP showed higher discriminatory power than *flaA*-RFLP	Wittwer *et al.* 2005
C. jejuni and *C. coli*	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	NS	Fang *et al.* 2006
C. jejuni, *Campylobacter helveticus*, *Campylobacter upsaliensis*	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	NS	Wieland *et al.* 2005
Campylobacter lari	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	NS	Desai *et al.* 2001a, Duim *et al.* 2004
Campylobacter spp.	*Hin*dIII – *Hha*I	Hin-A, Hha-A	F	AFLP showed better discriminatory power than PFGE	Keller *et al.* 2007
Clostridium botulinum	*Eco*RI – *Mse*I	Eco-T, Mse-T	F	AFLP was more discriminative than 16S rRNA gene sequencing	Hill *et al.* 2007

Table 1. Continued.

Species	Restriction enzyme combination	Primer combination in selective amplification [a]	F, R or S [b]	Comments on discriminatory power [c]	Reference
C. botulinum	*Eco*RI – *Mse*I	Eco-T, Mse-T	F	NS	Macdonald *et al*. 2008
Clostridium difficile	*Eco*RI – *Mse*I	Eco-0, Mse-G	F	Discriminatory power of AFLP comparable with that of PFGE	Klaassen *et al*. 2002
C. difficile	*Eco*RI – *Mse*I	Eco-0, Mse-G	F	AFLP had higher or equal discriminatory power with two different PCR ribotyping methods	Van den Berg *et al*. 2004
C. difficile	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	Discriminatory power of AFLP (D=0.631) lower than that of MLST (D=0.699), PCR-ribotyping (D=0.688), *slp*AST (D=0.815), PFGE (D=0.843), REA (D=0.933) and MLVA (D=0.964)	Killgore *et al*. 2008
Enterococcus faecium	*Hind*III – *Mbo*I	Hin-0, Mbo-AC and Hin-0, Mbo-CTG	F	AFLP and PFGE showed equal discriminatory power	Antonishyn *et al*. 2000
E. faecium	*Eco*RI – *Cfo*I	Eco-A, Cfo-G	F	Degree of strain differentiation by AFLP comparable with that of PFGE	Willems *et al*. 2000b
E. faecium	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	Discriminatory power of AFLP (D=0.75) lower than that of PFGE (D=0.89)	D'Agata *et al*. 2001
E. faecium	*Eco*RI – *Cfo*I	Eco-A, Cfo-G	F	AFLP showed higher discriminatory power than ribotyping and MLST	Brisse *et al*. 2002
E. faecium	*Eco*RI – *Cfo*I	Eco-A, Cfo-G	F	Degree of strain differentiation by MLST was comparable with that obtained by AFLP	Homan *et al*. 2002
E. faecium	*Hin*dIII – *Mse*I	Hin-A, Mse-A; Hin-A, Mse-C	F	NS	Vancanneyt *et al*. 2002
E. faecium	*Eco*RI – *Cfo*I	Eco-A, Cfo-G	F	AFLP and PFGE equally discriminative	Jureen *et al*. 2004
Enterococcus spp.	*Hin*dIII – *Mbo*I	Hin-C, Mbo-C	F	NS	Burtscher *et al*. 2006
Escherichia coli	*Eco*RI – *Mse*I	Eco-0, Mse-TA	F	AFLP groupings in concordance with groupings obtained by MLEE	Arnold *et al*. 1999b
E. coli	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	AFLP showed almost the same discriminating ability as PFGE	Iyoda *et al*. 1999
E. coli	*Eco*RI – *Mse*I	Eco-A, Mse-G	F	AFLP more discriminative than 16S rRNA gene sequencing	Guan *et al*. 2002

Table 1. Continued.

Species	Restriction enzyme combination	Primer combination in selective amplification [a]	F, R or S [b]	Comments on discriminatory power [c]	Reference
E. coli (shigatoxin-producing)	*Eco*RI – *Mse*I	Eco-0, Mse-C	F	PFGE (D=0.985) showed higher discriminatory power than AFLP (D=0.925)	Heir *et al*. 2000
E. coli O157:H7	*Eco*RI – *Mse*I	Eco-0, Mse-CG; Eco-A, Mse-CA; Eco-C,-Mse-C	F	AFLP more discriminatory than PFGE	Zhao *et al*. 2000
E. coli O157 (verocytotoxin-producing)	*Eco*RI – *Mse*I	Eco-0, Mse-TA, Mse-TC, Mse-TG and Mse-TT	F	AFLP and PFGE showed equal discriminatory power	Smith *et al*. 2000
Francisella tularensis	*Eco*RI - *Mse*I	Eco-C, Mse-A; Eco-0, Mse-CA; Eco-A, Mse-C and Eco-T, Mse-T	F	AFLP showed higher discriminatory power than 16S rRNA gene sequencing and lower discriminatory power than PFGE	García Del Blanco *et al*. 2002
Klebsiella pneumoniae	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	NS	Van der Zwet *et al*. 1999
Lactobacillus spp.	*Hin*dIII – *Mse*I	Hin-A, Mse-C; Hin-G, Mse-C	F	NS	Torriani *et al*. 2001
Leptospira interrogans	*Eco*RI – *Mse*I	6 different primer combinations used	F	NS	Vijayachari *et al*. 2004
L. interrogans	*Eco*RI – *Mse*I	6 different primer combinations used	F	AFLP showed lower discriminatory power than MLVA	Slack *et al*. 2006
Listeria monocytogenes	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	NS	Aarts *et al*. 1999
L. monocytogenes	*Eco*RI – *Bam*HI	Eco-0 , Bam-0	F	AFLP and PFGE showed equal discriminatory power	Fonnesbech Vogel *et al*. 2001
L. monocytogenes	*Eco*RI – *Bam*HI	Eco- 0, Bam-0	F	AFLP showed higher discriminatory power (D=0.974) than PFGE (D=0.969), RAPD (D=0.954) and ribotyping (D=0.874)	Fonnesbech Vogel *et al*. 2004
L. monocytogenes	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	AFLP showed higher discriminatory power than PCR-REA	Mikasová *et al*. 2005b
L. monocytogenes	*Eco*RI – *Mse*I	Eco-G, Mse-G	F	AFLP (D=0.964) showed higher discriminatory power than ribotyping (D=0.821) and MLSSCP (D=0.830) and lower discriminatory power than PFGE (D=0.971)	Takahashi *et al*. 2007

Table 1. Continued.

Species	Restriction enzyme combination	Primer combination in selective amplification [a]	F, R or S [b]	Comments on discriminatory power [c]	Reference
Mycobacterium avium subsp. *paratuberculosis*	*Eco*RI – *Mse*I	Eco-C, Mse-0	F	NS	Motiwala *et al*. 2003
M. avium subsp. *paratuberculosis*	*Pst*I – *Mse*I	96 different primer combinations used	F	NS	O'Shea *et al*. 2004
M. avium subsp. *paratuberculosis*	*Eco*RI – *Mse*I	Eco-A, Eco-C and Eco-G, Mse-0	F	NS	Kiehnbaum *et al*. 2005
Mycobacterium bovis, *Mycobacterium tuberculosis* and *Mycobacterium ulcerans*	*Apa*I – *Taq*I	Apa-C, Taq-C	R	NS	Huys *et al*. 2000
M. tuberculosis	*Eco*RI – *Mse*I	Eco-A, Eco-C, Eco-G, and Eco-T, Mse-0	F	AFLP showed higher discriminatory power than IS*6110* typing	Goulding *et al*. 2000b
M. tuberculosis	*Eco*RI – *Mse*I	Eco-0, Mse-0	F	AFLP showed higher discriminatory power than IS*6110* typing	Sims *et al*. 2002
M. tuberculosis	*Eco*RI – *Mse*I	Eco-A, Eco-C, Eco-G and Eco-T, Mse-0	F	NS	Ahmed *et al*. 2003
M. tuberculosis	*Eco*RI – *Mse*I	Eco-0, Mse-C	F	Discriminatory power of AFLP higher than that of IS*6110*-RFLP	Ruiz *et al*. 2003
M. tuberculosis	*Mbo*I – *Taq*I, *Nla*I – *Taq*I and *Mae*II – *Nla*I	8 different primer combinations used for each enzyme coupling	R	NS	Van den Braak *et al*. 2004
Mycoplasma capricolum subsp. *capripneumoniae* and *Mycoplasma mycoides* subsp. *mycoides*	*Eco*RI – *Csp*6I	Eco-0, Csp-A	F	AFLP showed higher discriminatory power than PFGE	Kusiluka *et al*. 2001
Mycoplasma spp.	*Bgl*II – *Mfe*I	Bgl-0, Mfe-0	F	NS	Kokotovic *et al*. 1999
Neisseria meningitides	*Eco*RI – *Mse*I	Eco-T, Mse-0	F	AFLP showed higher discriminatory power than PFGE	Goulding *et al*. 2000a

Table 1. Continued.

Species	Restriction enzyme combination	Primer combination in selective amplification [a]	F, R or S [b]	Comments on discriminatory power [c]	Reference
Pasteurella multocida	*Eco*RI – *Mse*I	Eco-A, Mse-0	F	Discriminatory power of AFLP (D=0.93) higher than that of rep-PCR (D=0.89)	Amonsin *et al*. 2002
P. multocida	*Eco*RI – *Mse*I	5 different primer combinations used	F	AFLP showed higher discriminatory power than RAPD	Huber *et al*. 2002
Pseudomonas aeruginosa	*Eco*RI – *Mse*I	Eco-0, Mse-0	F	AFLP showed higher discriminatory power than PFGE and RAPD	Speijer *et al*. 1999
P. aeruginosa	*Eco*RI – *Mse*I	Eco-A, Mse-0	F	Discriminatory power of AFLP (D=0.97) was higher than that of PFGE (D=0.96)	D'Agata *et al*. 2001
Pseudomonas spp.	*Eco*RI – *Mse*I	Eco-A, Mse-C	S	NS	Geornaras *et al*. 1999
Salmonella enterica	*Eco*RI – *Mse*I	Eco-0 , Mse-C	F	AFLP and PFGE showed equal discriminatory power	Lindstedt *et al*. 2000a
S. enterica	*Bgl*II – *Bsp*DI	Bgl-0 , Bsp-0	F	AFLP showed higher discriminatory power than MLST and similar discriminatory power to PFGE	Torpdahl *et al*. 2005
S. enterica serovar Abortusequi	*Eco*RI – *Mse*I	Eco-0, Mse-T	F	AFLP and PFGE showed equal discriminatory power	Akiba *et al*. 2003
S. enterica serovar Enteritidis	*Eco*RI – *Mse*I	Eco-0, Mse-C	F	Discriminatory power of AFLP (D=0.98) higher than that of PFGE (D=0.47)	Desai *et al*. 2001b
S. enterica serovar Enteritidis	*Eco*RI – *Mse*I	Eco-0, Mse-T and Eco-0, Mse-TA	F	AFLP showed higher discriminatory power than PFGE	Scott *et al*. 2001
S. enterica serovar Typhimurium	*Eco*RI – *Mse*I	Eco-A, Mse-A	F	PFGE showed higher discriminatory power than AFLP	Tamada *et al*. 2001
S. enterica serovar Typhimurium	*Eco*RI – *Mse*I	Eco-A, Mse-0	F	NS	Gebreyes and Altier 2002
S. enterica serovar Typhimurium	*Eco*RI – *Mse*I	16 different primer combinations used	F, R	NS	Hu *et al*. 2002
S. enterica serovar Typhimurium	*Eco*RI – *Bam*HI	Eco-0, Bam-0	F	AFLP showed lower discriminatory power than VNTR	Lindstedt *et al*. 2003

Table 1. Continued.

Species	Restriction enzyme combination	Primer combination in selective amplification [a]	F, R or S [b]	Comments on discriminatory power [c]	Reference
S. enterica serovar Typhimurium	*Hin*dIII – *Hha*I	Hind-0, Hha-C	F	AFLP showed higher discriminatory power than PFGE	Lawson *et al*. 2004
S. enterica serovar Typhimurium	*Eco*RI – *Mse*I	Eco-A, Mse-C	F	AFLP showed higher discriminatory power than PCR phage typing and detection of integrons	Mikasová *et al*. 2005a
S. enterica serovar Typhimurium	*Eco*RI – *Mse*I	Eco-A, Mse-0	F	Discriminatory power of AFLP (D=0.939) higher than that of PFGE (D=0.925) and rep-PCR (D=0.421)	Gebreyes *et al*. 2006
Salmonella spp.	*Eco*RI – *Mse*I	Eco-AA, Mse-0	R	NS	Aarts *et al*. 1998
Salmonella typhi	*Eco*RI – *Mse*I	18 different primer combinations were used	F	AFLP (D=0.88) showed higher discriminatory power than PFGE (D=0.74) and ribotyping (D=0.63)	Nair *et al*. 2000
Shigella flexneri and *Shigella sonnei*	*Eco*RI – *Mse*I	Eco-A, Mse-C; Eco-G, Mse-A; Eco-G, Mse-C	F	NS	Sirisriro *et al*. 2006
Staphylococcus aureus	*Mbo*I – *Csp*6I	Mbo-C and Mbo-G, Csp-TA	R	AFLP showed higher discriminatory power than MLST	Melles *et al*. 2007
S. aureus (methicillin-resistant)	*Eco*RI – *Mse*I and *Apa*I – *Taq*I	Eco-0, Mse-C Apa-0, Taq-G	F	Discriminatory power of AFLP was higher than that of PCR-RFLP and equal to that of PFGE	Grady *et al*. 1999
S. aureus (methicillin-resistant)	*Eco*RI – *Mse*I	Eco-0, Mse-AT	F	AFLP showed higher discriminatory power than RFLP and PFGE	Hookey *et al*. 1999
S. aureus (methicillin-resistant)	*Eco*RI – *Mse*I	Eco-0, Mse-C	F	Discriminatory power of PFGE higher than that of AFLP	Ip *et al*. 2003
S. aureus (methicillin-resistant)	*Eco*RI – *Mse*I	Eco-A, Eco-C and Eco-G, Mse-C	F	NS	Imataki *et al*. 2006
Staphylococcus epidermidis	*Eco*RI – *Mse*I	Eco-0, Mse-A	R	Discriminatory power of AFLP equal to that of antibiogram typing and PFGE and higher than that of biotyping, plasmid typing and RAPD	Sloos *et al*. 1998, Sloos *et al*. 2000

Table 1. Continued.

Species	Restriction enzyme combination	Primer combination in selective amplification [a]	F, R or S [b]	Comments on discriminatory power [c]	Reference
Staphylococcus spp.	*Hin*dIII – *Mse*I	Hind-G, Mse-C	F	NS	Taponen *et al*. 2007
Streptococcus pneumoniae	*Hin*dIII – *Taq*I	Hin-A, Taq-A	F	Discriminatory power of AFLP lower than that of PFGE	Van Eldere *et al*. 1999
S. pneumoniae	*Eco*RI – *Mse*I	Eco-A, Mse-0	F	PFGE showed higher discriminatory power (D=0.99) than AFLP (D=0.95)	Shaaly *et al*. 2005
Streptococcus pyogenes	*Eco*RI – *Mse*I	Eco-0, Mse-T	F	Discriminatory power of AFLP higher than that of PFGE	Desai *et al*. 1998
Streptococcus spp.	*Eco*RI - *Mse*I	Eco-0, Mse-G	F	Discriminatory power of AFLP comparable with that of PFGE	Neeleman *et al*. 2004
Streptococcus spp.	*Hin*dIII - *Mbo*I	Hind-C, Mbo-C	F	NS	Burtscher *et al*. 2006
Vibrio cholerae	*Eco*RI – *Mse*I	16 different primer combinations used	R	Discriminatory power of AFLP higher than that of RAPD	Lan and Reeves 2002
V. cholerae	*Apa*I – *Taq*I	Apa-A, Taq-A	F	NS	Thompson *et al*. 2003
Vibrio viscosus and *Vibrio wodanis*	*Hin*dIII - *Taq*I	Hin-A, Taq-A	R	NS	Benediktsdóttir *et al*. 2000
Vibrio spp.	*Hin*dIII - *Taq*I	Hin-A, Taq-G	F	NS	Thompson *et al*. 2001
Yersinia enterocolitica	*Bam*HI - *Bsp*DI	Bam-C, Bsp-T	F	NS	Fearnley *et al*. 2005
Y. enterocolitica, *Yersinia intermedia*, *Yersinia bercovieri*	*Bam*HI - *Bsp*DI	Bam-C, Bsp-T	F	NS	Kuehni-Boghenbor *et al*. 2006

[a] If several primer combinations initially tested, the combination showing the highest polymorphism is reported. A, C, G or T, selective nucleotide at the 3' end of the primer; 0, no selective nucleotide

[b] F, fluorescent labelling; R, radioactive labelling; S, silver staining

[c] AFLP, amplified fragment length polymorphism; ARDRA, amplified ribosomal DNA restriction analysis; D, discrimination index (Hunter and Gaston 1988); MLEE, multilocus enzyme electrophoresis; MLSSCP, multilocus single-strand conformation polymorphism; MLST, multilocus sequence typing; MLVA, multiple-locus variable-number tandem repeat analysis; NS, not studied; PCR-REA, polymerase chain reaction and restriction enzyme analysis; PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; rep-PCR, repetitive sequence-based PCR; RFLP, restriction fragment length polymorphism; *slp*AST, surface layer protein A gene sequence typing; VNTR, variable number tandem repeat

2.3 Modifications of the original AFLP method

2.3.1 Single-enzyme AFLP

In single-enzyme AFLP (sAFLP), the DNA is digested using one restriction enzyme (Valsangiacomo *et al*. 1995). Rare-cutter *Hin*dIII is commonly chosen (Table 2). Following digestion, restriction site-specific adapter is ligated to the fragments and PCR amplification using a single unlabelled primer is performed (Valsangiacomo *et al*. 1995). Most established protocols utilize primers having one selective nucleotide addition, but longer extensions have also been used (Table 2). Boumedine and Rodolakis (1998) applied a combination of seven different primers with three additional nucleotides to type *Chlamydia psittaci*. The resulting AFLP patterns were easy to interpret since only a few bands were obtained. However, the drawback of this protocol was that seven separate PCR amplifications had to be performed to achieve moderate discriminatory power. Another modification of sAFLP was established by Giammanco *et al*. (2007), who combined four primers in a single PCR reaction.

After PCR amplification, the fragments are separated by conventional horizontal gel electrophoresis on agarose gel and stained with ethidium bromide (Valsangiacomo *et al*. 1995, Gibson *et al*. 1999, De Zoysa and Efstratiou 2000, Ripabelli *et al*. 2000a, McLauchlin *et al*. 2002, Brett *et al*. 2005, Jaimes *et al*. 2006). A modification using 6% polyacrylamide gel and ethidium bromide staining has been described by Velappen *et al*. (2001). Fragments in a suitable size range are selected for numerical analysis. Typically, fragments smaller than 200-400 bp and larger than 1300-2000 bp are removed from the analysis (De Zoysa and Efstratiou 2000, McLauchlin *et al*. 2000, Ripabelli *et al*. 2000b, Champion *et al*. 2002, Guerra *et al*. 2002, Gaafar *et al*. 2003, Jaimes *et al*. 2006). The number of analysed fragments varies between 3 and 33 (Gibson *et al*. 1999, De Zoysa and Efstratiou 2000, Ripabelli *et al*. 2000a, 2000b, McLauchlin *et al*. 2000, 2002, Champion *et al*. 2002, Boerema *et al*. 2006, Jaimes *et al*. 2006, Rehm *et al*. 2007).

Compared with the original AFLP method, sAFLP generates significantly fewer fragments, therefore yielding less genetic information. However, the requirement for an automated sequencer may limit the use of fAFLP to reference and research laboratories, and therefore, sAFLP may be more widely applicable (Boerema *et al*. 2006). In sAFLP, amplified fragments are detected directly on agarose gel, making sAFLP easier to perform than, for example, some protocols of RFLP analysis (Valsangiacomo *et al*. 1995), ribotyping or insertion sequence typing (McLauchlin *et al*. 2000). In addition, sAFLP is easy to perform and the equipment required is inexpensive and widely available compared with that needed for PFGE (Ripabelli *et*

al. 2000a, Champion *et al*. 2002). sAFLP is also less labour-intensive and requires far less hands-on time than PFGE (Champion *et al*. 2002). Moreover, the sAFLP method is fast; results can be obtained within 24 h (Velappen *et al*. 2001, Boerema *et al*. 2006). If a limited number of sAFLP profiles is compared, *e.g.* in an outbreak situation, the patterns can be analysed visually (Ripabelli *et al*. 2000a, Velappen *et al*. 2001). However, computer-assisted analysis is needed when comparisons over extended time periods are performed (Ripabelli *et al*. 2000a).

Jonas *et al*. (2000) detected sAFLP products of *Legionella pneumophila* strains by using both an automated sequencer and agarose gel electrophoresis. Agarose gel electrophoresis gave additional information since fragments of more than 1000 bp, which could not be detected under the denaturing sequencing gel conditions used, were included in the analysis. However, analysis using an automated sequencer was convenient, allowed accurate definition of fragment size and was superior in detection of smaller fragments. In the same study, the discriminatory power of sAFLP was found to be higher than that of arbitrarily primed PCR, whereas PFGE identified a larger number of different genotypes than sAFLP. Champion *et al*. (2002) noted that sAFLP was equally discriminatory as PFGE when outbreaks of enteritis caused by campylobacters were investigated. PFGE and sAFLP showed similar discrimination also in a study of *Helicobacter pullorum* strains (Gibson *et al*. 1999). However, with 19 strains of *Salmonella enterica* serovar Typhimurium, PFGE identified 19 different patterns, while sAFLP generated only eight profiles (Sood *et al*. 2002). Similarly, in a study of *Staphylococcus aureus*, the discriminatory power of PFGE proved to be higher than that of sAFLP (Boerema *et al*. 2006). The modified sAFLP method using a mix of four primers in a single PCR reaction also failed to reach higher discriminatory power than PFGE when *S. enterica* serovar Enteritidis strains were genotyped (Giammanco *et al*. 2007).

Table 2. Single-enzyme AFLP protocols established for typing of bacteria.

Species	Restriction enzyme	Primer(s) used[a]	Reference
Bacillus anthracis, Bacillus cereus	*Hin*dIII	Hind-A, Hind-C, Hind-G, Hind-T, Hind-AC[b], Hind-AG, Hind-AA, Hind-AT, Hind-CA, Hind-CT, Hind-GA, Hind-GT, Hind-TA, Hind-TC, Hind-TG, Hind-TT	Velappen *et al*. 2001
B. cereus	*Hin*dIII	Hind-A[b], Hind-C, Hind-G, Hind-T	Ripabelli *et al*. 2000a
	*Hin*dIII	Hind-A	McLauchlin *et al*. 2002
Bacillus mycoides	*Hin*dIII	Hind-CA	Velappen *et al*. 2001
Bacillus thuringiensis	*Hin*dIII	Hind-CA	Velappen *et al*. 2001
Campylobacter jejuni	*Hin*dIII	Hind-C	Champion *et al*. 2002
Chlamydia psittaci	*Msp*I	Msp-CCT, Msp-CCA, Msp-GGT, Msp-CTA, Msp-ACT, Msp-CTC, Msp-GAA	Boumedine and Rodolakis 1998
Clostridium botulinum	*Hin*dIII	Hind-A, Hind-C[b], Hind-G, Hint-T	Brett *et al*. 2005
Clostridium novyi	*Hin*dIII	Hind-A, Hind-C, Hind-G, Hind-T	McLauchlin *et al*. 2002
	*Eco*RI	Eco-A, Eco-C, Eco-G, Eco-T	
Clostridium perfringens	*Hin*dIII	Hind-A, Hind-C, Hind-G[b], Hind-T	McLauchlin *et al*. 2000
	*Hin*dIII	Hind-G	McLauchlin *et al*. 2002
Clostridium spp.	*Hin*dIII	Hind-C	Jaimes *et al*. 2006
Corynebacterium diphteriae	*Pst*I	Pst-A, Pst-C, Pst-G[b], Pst-T	De Zoysa and Efstratiou 2000
Escherichia coli	*Hin*dIII	Hind-AC	Velappen *et al*. 2001
Helicobacter pullorum	*Hin*dIII	Hind-A, Hind-C[b], Hind-G[b], Hind-T	Gibson *et al*. 1999
Helicobacter pylori	*Hin*dIII	Hind-A[b], Hind-C, Hind-G, Hind-T	Gibson *et al*. 1998
Legionella pneumophila	*Pst*I	Pst-G, Pst-GC, Pst-A, Pst-AT	Valsangiacomo *et al*. 1995
	*Psti*I	Pst-G	Jonas *et al*. 2004
Listeria monocytogenes	*Hin*dIII	Hind-A[b], Hind-C, Hind-G, Hind-T	Ripabelli *et al*. 2000b
	*Eco*RI	Eco-0, Eco-A, Eco-C, Eco-G[b], Eco-T	Guerra *et al*. 2002
	*Eco*RI	Eco-G	Corcoran *et al*. 2006

Table 2. Continued.

Species	Restriction enzyme	Primer(s) used[a]	Reference
Mycobacterium kansasii	*Pst*I	Pst-GC, Pst-ATTAG	Picardeau *et al*. 1997
	*Apa*I	Apa-A[b], Apa-C[b], Apa-G, Apa-T[b]	Gaafar *et al*. 2003
Salmonella enterica serovar Enteritidis	*Hin*dIII	Hind-A, Hind-C, Hind-G, Hind-T[c]	Giammanco *et al*. 2007
Salmonella enterica serovar Havana	*Pst*I	Pst-A, Pst-G	Reche *et al*. 2003
Salmonella enterica serovar Typhimurium	*Hin*dIII	Hind-A, Hind-C[b], Hind-G, Hind-T	Sood *et al*. 2002
Shigella flexneri	*Pst*I	Pst-A[b], Pst-C, Pst-G, Pst-T	Herrera *et al*. 2002
Staphylococcus aureus	*Hin*dIII	Hind-AC	Velappen *et al*. 2001
	*Hin*dIII	Hind-A, Hind, C, Hind-G[b], Hind-T	Boerema *et al*. 2006
Streptococcus suis	*Hin*dIII	Hind-A, Hind-C, Hind-G[b], Hind-T	Rehm *et al*. 2007
Yersinia enterocolitica, *Yersinia pestis*, *Yersinia pseudotuberculosis*	*Hin*dIII	Hind-AC, Hind-G	Velappen *et al*. 2001

[a] A, C, G or T, selective nucleotide at the 3' end of the primer; 0, no selective nucleotide
[b] Reported to be the most suitable primer for AFLP analysis
[c] A mix of four primers used in a single PCR reaction

2.3.2 Other modified AFLP methods

If complete genome sequence of a bacterial strain is available, it can be used to predict which DNA fragments are amplified during AFLP analysis. *In silico* AFLP analysis of *Escherichia coli* showed that 97% of the predicted fragments were observed during AFLP analysis (Arnold *et al*. 1999b). Similarly, all but one of the 61 predicted fragments were detected when a strain of *Campylobacter jejuni* was studied (Desai *et al*. 2001a). However, when AFLP was applied for the G+C-rich genome of *Mycobacterium tuberculosis*, many predicted fragments were not observed. Because of the rich G+C content, secondary structures may be formed, which may cause incomplete digestion or poor amplification of fragments (Sims *et al*. 2002). The whole genome sequence information can also be used to screen for the most suitable restriction enzyme and primer combination, thus reducing the number of initial experiments needed to find a suitable coupling (Arnold *et al*. 1999b, Ahmed *et al*. 2003, Rombauts *et al*. 2003, Bikandi *et al*. 2004, Burtscher *et al*. 2006). Specific software programs have been developed for *in silico* AFLP analysis, and in addition

to the initial experiment design, they can be used for identification of the amplified fragments (Rombauts *et al*. 2003, Bikandi *et al*. 2004).

In three-endonuclease AFLP (TE-AFLP), the total genomic DNA is digested using one frequent-cutter and two rare-cutter restriction enzymes. Adapters are ligated only to ends generated by rare-cutters, and thus, the number of amplified fragments is lower than in the original AFLP protocol. The TE-AFLP method is especially suitable for the analysis of complex genomes such as plant or insect genomes (van der Wurff *et al*. 2000). A variation of the AFLP technique using one frequent-cutter and two rare-cutter enzymes and a mixture of three different primers during amplification has also been successfully applied to characterize *S. enterica* subsp. *enterica* isolates (Lindstedt *et al*. 2000b).

Van der Zee *et al*. (2003) developed a multi-enzyme multiplex-PCR AFLP. This method utilizes four different restriction enzymes. After digestion and ligation of adapters, two primers are used to amplify fragments, which are then separated by agarose gel electrophoresis. The discriminatory power of multi-enzyme multiplex-PCR AFLP is higher than that of sAFLP, and since an automated sequencer is not needed the method is suitable for routine use in clinical microbiology laboratories.

In single-adapter AFLP, one adapter is ligated to the cohesive ends generated by both restriction enzymes, circularizing the DNA fragments (Bootsma *et al*. 2000, Willems *et al*. 2000b). This method is, however, not widely used.

The first step of the complementary DNA (cDNA)-AFLP technique involves reverse transcription of messenger RNA into double-stranded cDNA. The double-stranded cDNA is then digested with two restriction enzymes, followed by ligation of adapters and PCR amplification steps (Dellagi *et al*. 2000, Kivioja *et al*. 2005). The resulting amplification products are separated by polyacrylamide gel electrophoresis (Dellagi *et al*. 2000, Breyne *et al*. 2003). Gene expression profiles can then be determined by quantitative analysis of band intensities (Breyne *et al*. 2003). The method also allows identification of differentially expressed genes if amplified cDNA products are purified from gels and sequenced (Dellagi *et al*. 2000). Since cDNA-AFLP enables expression analysis without the need for prior sequence knowledge, it can be used as an alternative to microarrays, especially when genome sequence information is limited (Breyne *et al*. 2003, Reijans *et al*. 2003).

AFLP analysis can also be used to identify specific markers, such as species-specific fragments, which can then be excised from the AFLP gel, reamplified by PCR and sequenced (Tamada *et al*. 2001, Hu *et al*. 2002, van den Braak *et al*. 2004, van Bergen *et al*. 2005b). The resulting sequences can be utilized in development of PCR-based diagnostic assays, *e.g.* species-specific PCRs (van Bergen *et al*. 2005b).

2.4 AFLP pattern analysis

Differences in AFLP patterns of different strains arise from insertions or deletions within the amplified fragments and from mutations in the restriction sites or in the sequences adjacent to the restriction sites and complementary to the selective primer extensions (Savelkoul *et al*. 1999). The choice of a suitable restriction enzyme and primer combination is important to achieve a sufficient number of polymorphic bands (Valsangiacomo *et al*. 1995, Lan and Reeves 2002). Typically, the fragment sizes included in pattern analysis vary between 50 and 500 bp (Aarts *et al*. 1999, Heir *et al*. 2000, Lindstedt *et al*. 2000c, On and Harrington 2000, Willems *et al*. 2000b, Kusiluka *et al*. 2001, Amonsin *et al*. 2002, Guan *et al*. 2002, Motiwala *et al*. 2003, Shaaly *et al*. 2005, Torpdahl *et al*. 2005, Hill *et al*. 2007).

There are several ways to perform AFLP pattern analysis. The two most commonly used techniques are to calculate percentage similarities between AFLP patterns using Dice correlation coefficient (Dice 1945, Hookey *et al*. 1999, Scott *et al*. 2001, Lan and Reeves 2002, Sawabe *et al*. 2002, Ip *et al*. 2003, Motiwala *et al*. 2003, Mikasová *et al*. 2005, Torpdahl *et al*. 2005) or Pearson product-moment correlation coefficient (Pearson 1926, Duim *et al*. 1999, van Eldere *et al*. 1999, De Boer *et al*. 2000, van der Zwet *et al*. 2000, Hänninen *et al*. 2001, Schouls *et al*. 2003, van der Zee *et al*. 2003, On *et al*. 2004, van den Berg *et al*. 2004, Fearnley *et al*. 2005, Wieland *et al*. 2005, Burtscher *et al*. 2006, Fang *et al*. 2006, Kuehni-Boghenbor *et al*. 2006, Keller *et al*. 2007, Takahashi *et al*. 2007). Dice correlation coefficient is based on band presence or absence, and therefore, band assignment is necessary (De Boer *et al*. 2000), whereas Pearson product-moment correlation coefficient measures the whole densitometric curve of the gel track without assignment of bands (De Boer *et al*. 2000, van der Zee *et al*. 2003).

A drawback of band-based analysis is that the band assignment can be very laborious due to complex AFLP patterns (De Boer *et al*. 2000, Werner *et al*. 2003). In addition, band-based analysis is subject to human interpretation errors, and various parameters, such as different technicians, presence of bands that have very similar sizes and gel electrophoresis conditions, can affect the outcome (Duim *et al*. 2000, van der Zee *et al*. 2003, Torpdahl *et al*. 2005). Potential person-to-person variation can make comparison of the results of band-based AFLP analysis between laboratories difficult (Torpdahl *et al*. 2005). On the other hand, the Pearson correlation coefficient method is sensitive to differences in background and to lesser extent to variations in relative band intensities (De Boer *et al*. 2000, Huys *et al*. 2000, Werner *et al*. 2003). Especially if manual sequence equipment and radioactively labelled primers are used, standardization of the background intensity can be challenging (Coenye *et al*. 1999b, De Boer *et al*. 2000). It also is essential to

remember that although computer software is partly automated the selected settings affect the outcome of both analyses (Gerner-Smidt *et al*. 1998, De Boer *et al*. 2000, Fry *et al*. 2000). The settings selected should thus be kept constant throughout the study (De Boer *et al*. 2000).

Duim *et al*. (2000) analysed AFLP banding profiles of *C. jejuni* strains with band-based and correlation-based similarity coefficients, and construction of dendrograms was performed by the unweighted pair-group method using average linkages. Analyses resulted in dendrograms with identical clustering and discrimination of strains. Werner *et al*. (2003), by contrast, found that the level of similarities of AFLP profiles of *Enterococcus faecium* strains and the resulting cluster analysis were strongly dependent on the coefficient used for calculating similarities.

2.5 Strengths and weaknesses of AFLP

AFLP can be applied to DNA of any origin and complexity, and no prior knowledge about the target DNA is needed (Vos *et al*. 1995). Furthermore, AFLP analysis reflects the total genome of the organism and the amplified fragments originate from both variable and conserved DNA sequences (Arnold *et al*. 1999a, Willems *et al*. 2000b). Thus, the method is considered to provide knowledge of the short- and long-term evolution of bacterial strains (Thompson *et al*. 2003). The discriminatory power of AFLP has been shown to be high in several studies (Table 1). A standard set of reagents can be applied to different bacterial species without the need for species-specific information (Jackson *et al*. 1999, van der Zwet *et al*. 2000). However, an AFLP protocol providing high discriminatory ability with one bacterial species may not necessarily yield good results when applied to another species (Lindstedt *et al*. 2000b). The discriminatory power of AFLP can be controlled by selection of the restriction enzyme and primer combination used (Desai *et al*. 1998, Heir *et al*. 2000, Mortimer and Arnold 2001), and it is therefore essential to tailor the protocol for optimal characterization of each bacterial species investigated.

AFLP analysis is relatively insensitive to differences in the concentrations of template DNA. Vos *et al*. (1995) have shown that template concentrations ranging 1000-fold, from 25 pg to 25 ng, had little effect on AFLP patterns. However, with a very low DNA concentration of 2.5 pg, differences in band intensity were observed and some bands were absent. Burtscher *et al*. (2006) found that AFLP profiles were stable at DNA concentrations of 0.1-100 ng for digestion. However, concentrations varying from 500 to 1500 ng resulted in intensity variations and a loss of longer fragments. Similarly, Gzyl *et al*. (2005) did not find any band variations in the AFLP profiles when DNA concentrations of 100–500 ng were used.

In contrast to RAPD, which has a low reproducibility (Power *et al*. 1996), AFLP uses highly stringent PCR conditions, and thus, its reproducibility is good. Several studies have reported that duplicate experiments resulted in identical AFLP patterns (Janssen *et al*. 1996, Koeleman *et al*. 1998, Sloos *et al*. 1998, Grady *et al*. 1999, Kokotovic *et al*. 1999, Antonishyn *et al*. 2000, Goulding *et al*. 2000a, Pattanayak *et al*. 2000, Smith *et al*. 2000, Kusiluka *et al*. 2001, Scott *et al*. 2001, Hu *et al*. 2002, Kassama *et al*. 2002). Many authors have, however, observed small variation in peak heights or fragment intensities (Duim *et al*. 1999, Jackson *et al*. 1999, Kokotovic and On 1999, Antonishyn *et al*. 2000, Sims *et al*. 2002, On *et al*. 2004, Hong *et al*. 2005). Variations in peak height have not affected the numbers or sizes of the PCR products. This variation is due to small differences in lane and background intensities or peak heights, which may arise from differences in the effectiveness of digestion-ligation or PCR amplification steps (Lindstedt *et al*. 2000a) as well as from minor differences in the amounts of sample loaded onto the gel (Ticknor *et al*. 2001). The degree of similarity of repeated AFLP experiments has varied between 84% and 99%, as determined by the Pearson product-moment correlation in different studies (Huys *et al*. 1996a, 2000, Janssen *et al*. 1997, Sloos *et al*. 1998, Duim *et al*. 1999, 2004, van Eldere *et al*. 1999, On and Harrington 2000, Willems *et al*. 2000a, Willems *et al*. 2000b, Vancanneyt *et al*. 2002, On *et al*. 2003, 2004, Fearnley *et al*. 2005, Kuehni-Boghenbor *et al*. 2006, Wieland *et al*. 2006, Keller *et al*. 2007, Rehm *et al*. 2007).

The major challenge of most typing methods, including AFLP analysis, is interlaboratory reproducibility (Brisse *et al*. 2002). Although several authors have suggested that AFLP profiles are suitable for electronic transmission for interlaboratory comparisons (Desai *et al* 1998, Duim *et al*. 1999, Antonishyn *et al*. 2000, De Boer *et al*. 2000, Heir *et al*. 2000, Mortimer and Arnold 2001, Tamada *et al*. 2001, Guan *et al*. 2002, van den Berg *et al*. 2004), only a few studies have examined this issue. Jones *et al*. (1997) found that when laboratories gained experience with the AFLP method utilizing radioactively labelled primers the AFLP profiles showed extremely high reproducibility. Similarly, when sAFLP was used to genotype *L. pneumophila* isolates, the results of the intercentre comparison were promising. However, several experimental parameters potentially can affect intercentre reproducibility (Fry *et al*. 2000, 2002). To create databases utilizing results from different laboratories, standardized AFLP protocols using identical instrumentation and reagents acquired from the same manufacturer must be established. In addition, criteria chosen to define the AFLP types must be selected carefully (Fry *et al*. 2000). Due to the wide selection of different automated sequencers currently available for AFLP analysis, fAFLP may prove to be difficult to standardize across laboratories.

PFGE is often considered to be the gold standard for bacterial typing (Heir *et al*. 2000, Klaassen *et al*. 2002, Ip *et al*. 2003, Shaaly *et al*. 2005). However, several studies have shown that the discriminatory power of AFLP is equal or higher than that obtained with PFGE (Table 1). AFLP analysis is faster than PFGE analysis, and the technique can be partly automated, thus reducing the required hands-on time and enabling high throughput of samples (Duim *et al*. 1999, Grady *et al*. 1999, Heir *et al*. 2000, Lindstedt *et al*. 2000a, Smith *et al*. 2000, Scott *et al*. 2001, Klaassen *et al*. 2002). In addition, some automated DNA sequencers allow the use of several fluorescent dyes simultaneously, and thus, the efficiency can be increased by running different AFLP reactions simultaneously (Heir *et al*. 2000). At present, the cost of an automated DNA sequencer may limit the routine application of AFLP analysis in clinical settings (Gaafar *et al*. 2003). The cost of a single AFLP reaction is, nevertheless, estimated to be lower than that of PFGE (Olive and Bean 1999). AFLP is also more cost-efficient and easier and faster to perform than MLST (Schouls *et al*. 2003).

AFLP allows more precise sizing of fragments (± 1 bp) than PFGE, and more fragments are available for comparison and definition of strain genotype (Desai *et al*. 1998, Smith *et al*. 2000, Tamada *et al*. 2001). A limited number of PFGE patterns can be compared to each other by eye, and computer software is generally required for comparison of more complex AFLP patterns (Klaassen *et al*. 2002, Jureen *et al*. 2004). Computer-assisted analysis also facilitates the processing of large numbers of samples and may enable transfer of data between different laboratories (De Boer *et al*. 2000).

One of the disadvantages of AFLP is that it is not possible to know whether identically sized fragments are derived from the same part of the genome (Gibson *et al*. 1998). In addition, if specific characterization is needed, the fragments must to be sequenced after DNA extraction from capillary electrophoresis fractions or gel bands (Hu *et al*. 2002, Rombauts *et al*. 2003). This can be difficult if fAFLP is used (Portier *et al*. 2006). However, when a whole genome sequence is available, *in silico* AFLP analysis can aid in fragment identification (Rombauts *et al*. 2003, Bikandi *et al*. 2004).

2.6 Use of AFLP in species differentiation

The current consensus for bacterial species determination is based on whole-genome DNA-DNA hybridization analysis, with a species comprising strains showing at least 70% DNA-DNA reassociation and a $\Delta T_m \leq 5°C$ (difference in DNA-DNA hybrid melting points) (Wayne *et al*. 1987). However, this method is not practical for routine

use and is difficult to apply to a large number of isolates, and therefore, alternative approaches have been developed (Mougel *et al*. 2002). At present, 16S rRNA gene sequence analysis is widely used for species identification (Clarridge III 2004), and an international committee has recommended that all species descriptions should involve an almost complete 16S rRNA sequence (Stackebrandt *et al*. 2002). In general, organisms showing less than 97% 16S rRNA sequence identity will not give a DNA similarity of more than 60% and thus belong to different species (Stackebrandt and Goebel 1994). On the other hand, organisms showing over 97% 16S rRNA sequence similarity may belong to a single species. However, this is not always the case, and the approach is therefore insufficient to guarantee species identification (Fox *et al*. 1992, Stackebrandt and Goebel 1994, Vandamme *et al*. 1996). Recently, an international committee has suggested that determination of species can be obtained with alternative molecular methods to DNA-DNA hybridization analysis, describing also AFLP as a promising method (Stackebrandt *et al*. 2002).

The first report evaluating the applicability of AFLP in bacterial taxonomy was published in 1996 (Janssen *et al*. 1996). Since then, AFLP has been used to study species-level diversity in several groups of bacteria (Table 3). Huys *et al*. (1996) found a strong overall level of correlation between AFLP fingerprinting data of the genus *Aeromonas* and DNA-DNA hybridization results. Later, AFLP analysis has been shown in several studies to reflect well DNA-DNA similarity, *e.g.* in *Agrobacterium* (Mougel *et al*. 2002, Portier *et al*. 2006), *Burkholderia* (Coenye *et al*. 1999b), *Ralstonia* (Coenye *et al*. 1999a) and *Xanthomonas* (Rademaker *et al*. 2000). AFLP can thus be considered a relatively fast and reliable alternative to DNA-DNA hybridization studies and especially suitable for screening large number of isolates (Rademaker *et al*. 2000). However, ultimate confirmation should be achieved with DNA-DNA hybridization analysis (Janssen *et al*. 1997).

Duim *et al*. (2001) found that AFLP was a suitable tool for identification of *Campylobacter* strains at the species and subspecies levels. However, the AFLP dendrogram did not correspond with the phylogenetic relationships derived from 16S rRNA sequence comparison. For *Ralstonia* species, by contrast, the AFLP results were in agreement with 16S rRNA sequence analysis (Coenye *et al*. 1999a), and for streptococcal isolates AFLP analysis provided a more definite identification than 16S rRNA sequencing (Neeleman *et al*. 2004). Coenye *et al*. (1999b) have also shown that AFLP is a valuable or even a better alternative than SDS-PAGE of whole-cell proteins for distinguishing *Burkholderia* species.

Table 3. Studies that have used AFLP in bacterial species differentiation.

Species	References
Acinetobacter spp.	Janssen *et al*. 1997, Koeleman *et al*. 1998, Chang *et al*. 2005
Aeromonas spp.	Huys *et al*. 1996a, 1996b
Agrobacterium spp.	Mougel *et al*. 2002, Portier *et al*. 2006
Arcobacter spp.	On *et al*. 2003
Bacillus spp.	Keim *et al*. 1997, Ticknor *et al*. 2001, Hill *et al*. 2004
Bordetella spp.	Gzyl *et al*. 2005
Brucella spp.	Whatmore *et al*. 2005
Burkholderia spp.	Coenye *et al*. 1999b, 2001
Campylobacter spp.	On and Harrington 2000, Duim *et al*. 2001, Wieland *et al*. 2005, Keller *et al*. 2007, Waldenström *et al*. 2007
Citrobacter freundii, *Enterobacter* spp., *Enterococcus* spp., *Escherichia coli*, *Klebsiella* spp., *Morganella morganii*, *Providencia rettgeri*, *Proteus mirabilis*	Kassama *et al*. 2002
Enterococcus spp.	Burtscher *et al*. 2006
Erwinia spp.	Avrova *et al*. 2002
Lactobacillus spp.	Torriani *et al*. 2001
Mycobacterium spp.	Huys *et al*. 2000
Mycoplasma spp.	Hong *et al*. 2005
Ralstonia spp.	Coenye *et al*. 1999a
Staphylococcus spp. (coagulase-negative)	Taponen *et al*. 2006, 2007
Streptococcus spp.	Neeleman *et al*. 2004
Vibrio spp.	Benediktsdóttir *et al*. 2000, Thompson *et al*. 2001
Xanthomonas spp.	Rademaker *et al*. 2000
Yersinia spp.	Kuehni-Boghenbor *et al*. 2006

If a standardized AFLP protocol is used, AFLP data are suitable for creation of an identification library (Huys *et al*. 1996b, Janssen *et al*. 1997, Chang *et al*. 2005, Hong *et al*. 2005, Whatmore *et al*. 2005), which can aid in identifying unknown isolates of a particular bacterial genus. Waldenström *et al*. (2007) successfully utilized an AFLP identification library of *Campylobacter* strains for presumptive identification of campylobacteria isolated from wild birds. A polyphasic identification approach including 16S RNA sequence analysis and extensive phenotypic characterization could then be limited to selected strains.

2.7 Genus *Listeria*

2.7.1 Classification, characteristics and clinical significance of listeria

Listeria spp. are Gram-positive, non-sporing, facultatively anaerobic rods (Seeliger and Jones 1986) that are widely distributed in the environment, including soil, sewage and vegetation (Ramaswamy *et al*. 2007). Type species of the genus is *L. monocytogenes* and the mol% G+C of the DNA varies between 36 and 39 (Seeliger and Jones 1986, Glaser *et al*. 2001). The genus *Listeria* belongs to the family *Listeriaceae*, the order *Bacillales*, the class *Bacilli* and the division *Firmicutes* and contains six species: *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri* and *L. welshimeri* (Euzéby 1997, Vázquez-Boland *et al*. 2001). Two of the species, *L. monocytogenes* and *L. ivanovii*, are known to be pathogenic. *L. ivanovii* mainly causes disease in animals, but a few cases have also been reported in humans, whereas *L. monocytogenes* causes listeriosis in both animals and humans (Low and Donachie 1997, Vázquez-Boland *et al*. 2001).

2.7.2 *L. monocytogenes* and listeriosis

Healthy animals and humans can serve as non-symptomatic carriers of *L. monocytogenes*. However, *L. monocytogenes* can also cause a severe disease, invasive listeriosis, which has a fatality rate as high as 30%. Pregnant women, the elderly, newborns and immunocompromised persons are at higher risk of contracting invasive listeriosis (Farber and Peterkin 1991, Ramaswamy *et al*. 2007). Clinical manifestations of invasive listeriosis include abortion, stillbirth, sepsis, meningitis and meningoencephalitis (Vázquez-Boland *et al*. 2001, Ramaswamy *et al*. 2007). In otherwise healthy people, *L. monocytogenes* may cause non-invasive febrile gastroenteritis (Riedo *et al*. 1994, Dalton *et al*. 1997, Miettinen *et al*. 1999b, Sim *et al*. 2002). In addition, veterinarians and farmers are at higher risk of contracting cutaneous listeriosis by direct contact with infected animals (McLauchlin and Low 1994). Most listeriosis cases are foodborne; vegetables (Schlech *et al*. 1983, Aureli *et al*. 2000), dairy products (Fleming *et al*. 1985, Dalton *et al*. 1997, Lyytikäinen *et al*. 2000), ready-to-eat meat products (de Valk *et al*. 2001, Sim *et al*. 2002) and seafood (Brett *et al*. 1998, Miettinen *et al*. 1999b) have been implicated in listeriosis outbreaks.

2.7.3 *L. monocytogenes* in the food processing environment

Control of *L. monocytogenes* in food processing plants is extremely challenging since the organism survives and grows at refrigerated temperatures, tolerates high salt concentration and low pH and can exist in biofilms on surfaces of the food processing plant (Gandhi and Chikindas 2007). Various genotyping methods have been used to trace the sources of *L. monocytogenes* contamination in the food processing industry (Destro *et al*. 1996, Giovannacci *et al*. 1999, Chasseignaux *et al*. 2001, Fonnesbech Vogel *et al*. 2001, Berrang *et al*. 2002, Hu *et al*. 2006, De Cesare *et al*. 2007, López *et al*. 2007). Contamination routes of *L. monocytogenes* have been widely surveyed, especially in seafood (Rørvik *et al*. 1995, Destro *et al*. 1996, Autio *et al*. 1999, Johansson *et al*. 1999, Dauphin *et al*. 2001, Fonnesbech Vogel *et al*. 2001, Norton *et al*. 2001, Vaz-Velho *et al*. 2001, Hoffman *et al*. 2003, Lappi *et al*. 2004, Thimothe *et al*. 2004, Gudmundsdóttir *et al*. 2005, 2006, Hu *et al*. 2006, Nakamura *et al*. 2006, Wulff *et al*. 2006), dairy (Unnerstad *et al*. 1996, Miettinen *et al*. 1999a, Lyytikäinen *et al*. 2000, Wagner *et al*. 2006, De Cesare *et al*. 2007), meat (Nesbakken *et al*. 1996, Giovannacci *et al*. 1999, Autio *et al*. 2000, Chasseignaux *et al*. 2001, Lundén *et al*. 2003b, Heir *et al*. 2004, Thévenot *et al*. 2006a, 2006b, Bērziņš *et al*. 2007) and poultry processing plants (Lawrence and Gilmour 1995, Chasseignaux *et al*. 2001, Berrang *et al*. 2002, Lundén *et al*. 2003b, Rørvik *et al*. 2003, López *et al*. 2007).

Since *L. monocytogenes* is ubiquitous in nature, the initial contamination can be introduced to the food processing plant by several routes, including raw materials, personnel, transport vehicles, equipment and packaging materials (Lawrence and Gilmour 1995, Rørvik *et al*. 2000, Berrang *et al*. 2002, Lundén *et al*. 2002, Hoffman *et al*. 2003, Markkula *et al*. 2005, Wagner *et al*. 2006). Due to the inevitable presence of *L. monocytogenes* in the low-risk area where raw materials are handled, it is essential to have hygiene barriers that hinder the spread of the organism to areas requiring greater hygiene (Berrang *et al*. 2002, Heir *et al*. 2004, Gudmundsdóttir *et al*. 2006). However, incoming strains do not always contaminate the environment, and, in general, the *L. monocytogenes* population present in raw materials is different from the population persisting in the plant environment (Hoffman *et al*. 2003, Thimothe *et al*. 2004). The environment of the plant is often colonized by a few dominant clones (Rørvik *et al*. 1995, Autio *et al*. 1999, Giovannacci *et al*. 1999, Johansson *et al*. 1999, Miettinen *et al*. 1999a, Dauphin *et al*. 2001, Fonnesbech Vogel *et al*. 2001, Norton *et al*. 2001, Martinez *et al*. 2003, Rørvik *et al*. 2003, Holah *et al*. 2004, Wulff *et al*. 2006). The persistent strains seem to have characteristics, such as enhanced adherence to food contact surfaces, increased biofilm formation and resistance to disinfectants, that favour their persistence in the plant (Norwood and Gilmour 1999, Lundén *et al*.

2000, 2003a, Borucki *et al*. 2003b). Several studies have shown that *L. monocytogenes* can persist in food processing plants for prolonged periods of time, even several years, indicating that routine cleaning may fail to eliminate the organism (Nesbakken *et al*. 1996, Unnerstad *et al*. 1996, Giovannacci *et al*. 1999, Johansson *et al*. 1999, Miettinen *et al*. 1999a, Fonnesbech Vogel *et al*. 2001, Hoffman *et al*. 2003, Martinez *et al*. 2003, Thimothe *et al*. 2004, Hu *et al*. 2006, Wagner *et al*. 2006, Wulff *et al*. 2006).

Raw materials may represent the source of finished product contamination (Norton 2001, Hoffman 2003, Gudmundsdóttir *et al*. 2005, Markkula 2005). The role of raw materials is particularly important if the preparation process does not involve a listericidal heat treatment (Hoffman *et al*. 2003, López *et al*. 2007). However, most authors agree that the contamination of finished products seems primarily to arise from post-processing contamination from the environment (Rørvik *et al*. 1995, Nesbakken *et al*. 1996, Autio *et al*. 1999, Johansson *et al*. 1999, Miettinen *et al*. 1999a, Lyytikäinen *et al*. 2000, Rørvik *et al*. 2000, Dauphin *et al*. 2001, Fonnesbech Vogel *et al*. 2001, Lundén *et al*. 2003b, Thimothe *et al*. 2004, Nakamura *et al*. 2006), and contamination of the environment has been shown to increase along the processing line (Rørvik *et al*. 2003). Typically, the source of finished product contamination has been observed to be processing machines, particularly brining, slicing, dicing and packing machines (Autio *et al*. 1999, Johansson *et al*. 1999, Lyytikäinen *et al*. 2000, Dauphin *et al*. 2001, Fonnesbech Vogel *et al*. 2001, Lundén *et al*. 2002, Nakamura *et al*. 2006, Bērziņš *et al*. 2007). However, within a plant, the contamination can also be spread by other tools or personnel; *L. monocytogenes* has been recovered from, for instance, the hands, footwear, gloves and aprons of employees (Destro *et al*. 1996, Autio *et al*. 1999, Dauphin *et al*. 2001, Gudbjörnsdóttir *et al*. 2004, Thimothe *et al*. 2004, Gudmundsdóttir *et al*. 2005). Furthermore, job rotation between departments has been shown to be a risk factor associated with the isolation of *L. monocytogenes* from smoked salmon, and thus, the role of personnel can become considerably larger when assigned duties are rotated (Rørvik *et al*. 1997). Lundén *et al*. (2002) have also reported the transfer of persistent *L. monocytogenes* contamination between food processing plants with a dicing machine. Therefore, it is important to limit the traffic of staff and equipment to avoid cross-contamination between different processing lines, compartments and even food processing plants (Rørvik *et al*. 2000, Lundén *et al*. 2003b, Thimothe *et al*. 2004, Gudmundsdóttir *et al*. 2006). To avoid product contamination, efficient cleaning and disinfection routines are essential with special attention directed to processing machines and other product contact surfaces (Rørvik *et al*. 2000, Lundén *et al*. 2002, 2003b, Thimothe *et al*. 2004).

2.8 Genus *Clostridium*

2.8.1 Classification, characteristics and clinical significance of clostridia

The genus *Clostridium*, proposed by Prazmowski in 1880, belongs to the family *Clostridiaceae*, the order *Clostridiales*, the class *Clostridia* and the division *Firmicutes* (Cato *et al*. 1986, Euzéby 1997). This genus is one of the largest for bacteria (Collins *et al*. 1994), which at present contains 190 validly named species (Euzéby 1997). The genus *Clostridium* is phenotypically extremely heterogeneous, warranting a major taxonomic revision (Collins *et al*. 1994). Clostridia are Gram-positive, spore-forming, straight or slightly curved rods. Most *Clostridium* species are obligate anaerobes. There is, however, wide variety in oxygen tolerance, and some species, such as *C. histolyticum* and *C. tertium*, are able to grow in the presence of oxygen. Type species *C. butyricum* has a DNA base composition of 27-28 mol% G+C. Although the G+C content of the DNA for other *Clostridium* species varies between 22 and 55 mol% (Cato *et al*. 1986), most species have a low G+C content (Johnson and Francis 1975, Collins *et al*. 1994).

Clostridium species are ubiquitous in nature, existing primarily in soil, freshwater and marine sediments and the intestinal tract of humans and many animals (Goonetilleke and Harris 2004). Although most *Clostridium* species are harmless saprophytes, a few clostridia, the so-called major pathogens, are involved in a variety of serious and often fulminant human and animal diseases (Hatheway 1990) (Table 4). The illnesses caused by major pathogens are mediated by their toxins (Hatheway 1990). In addition to the major pathogens, severe disease can be caused by *C. baratii* and *C. butyricum* strains, which are able to produce type E or F botulinum toxin (Hatheway 1990, Wang *et al*. 2000, Barash *et al*. 2005). Moreover, many *Clostridium* species are opportunistic pathogens and can cause various clinical conditions such as soft tissue infections, abscesses, intra-abdominal infections, pleuropulmonary infections and bacteraemia (Cato *et al*. 1986, Lavigne *et al*. 2003). Recently emerging clostridia, which have been considered to be harmless and non-pathogenic, have also been implicated in severe human infections (Carlier *et al*. 2004, 2006, Elsayed and Zhang 2004, 2007, Woo *et al*. 2004, 2005). In addition, many older clostridial diseases, which were previously associated mainly with injuries resulting from warfare, have re-emerged and caused life-threatening conditions among injecting drug users (Brazier *et al*. 2002).

Table 4. Major pathogens of the genus *Clostridium*.

Species	Disease	Reference
C. botulinum	Foodborne, infant, wound and adult infectious botulism	Goonetilleke and Harris 2004, Lindström and Korkeala 2006
C. chauvoei	Blackleg in cattle and sheep	Hatheway 1990
C. colinum	Ulcerative enteritis and necrotizing hepatitis in fowl	Songer 1996
C. difficile	Antibiotic-associated diarrhoea, pseudomembranous colitis	Knoop *et al*. 1993
C. haemolyticum	Haemoglobinuria in cattle	Smith 1952
C. histolyticum	Wound infections, gas gangrene	Hatheway 1990, Brazier *et al*. 2004
C. novyi	Gas gangrene in humans, necrotic hepatitis in sheep	Hatheway 1990
C. perfringens	Food poisoning, necrotic enteritis, gas gangrene, antibiotic-associated diarrhoea in humans, gastrointestinal and enterotoxaemic diseases in animals	Rood and Cole 1991, Petit *et al*. 1999
C. piliforme	Tyzzer's disease in animals	Van Andel *et al*. 2000
C. septicum	Gas gangrene, enterocolitis, necrotic myositis, necrotic dermatitis	Smith-Slatas *et al*. 2006
C. sordellii	Wound and bone infections, bacteraemia, gas gangrene and fulminate endometritis in humans, enteritis and enterotoxaemia in sheep and cattle	Bitti *et al*. 1997, Lewis and Naylor 1998, Abdulla and Yee 2000, Sinave *et al*. 2002
C. spiroforme	Enterotoxaemia in rabbits and laboratory rodents	Borriello and Carman 1983, Songer 1996
C. tetani	Tetanus	Goonetilleke and Harris 2004

2.8.2 Identification of clostridia

Despite the clinical importance and food hygiene risk of clostridia, reliable, practical and fast identification methods are few. Traditionally, the identification of clostridia has been based on Gram-staining, morphology, biochemical testing and analysis of short-chain fatty acid metabolites of glucose fermentation by gas-liquid chromatography (Holdeman *et al*. 1977). These methods are time-consuming, laborious, expensive and sometimes fail to identify clostridia to the species level, and thus, are not applicable in many clinical or food microbiology laboratories (Celig and Schreckenberger 1991, Sperner *et al*. 1999a, Song *et al*. 2002, Elsayed and Zhang 2004). In addition, some clostridia may be misidentified due to Gram stain variability, lack of spores and atypical clostridial colonial morphology (Alexander *et al*. 1995, Lavigne *et al*. 2003, Elsayed and Zhang 2004).

Several commercial identification kits for anaerobic bacteria, including API AN-Ident, API 20A, ATB 32A, Minitek Anaerobe II, PRAS II, Rapid ID 32 A, RapID

ANA II and Vitek ANI, have failed to accurately identify *Clostridium* species, and none of these kits can thus be used as the sole identification method for clostridia (Burlage and Ellner 1985, Head and Ratnam 1988, Kitch and Appelbaum 1989, Looney *et al*. 1990, Celig and Schreckenberger 1991, Marler *et al*. 1991, Alexander *et al*. 1995, Lindström *et al*. 1999, Sperner *et al*. 1999a, Lau *et al*. 2006). Therefore, various genotypic identification methods, such as 16S-23S rDNA internal transcribed spacer (ITS) polymorphism analysis, PCR-RFLP targeting triosephosphate isomerise (*tpi*) gene and automated ribotyping, have been tested (Broda *et al*. 2003, Dhalluin *et al*. 2003, Kennet and Stark 2006). However, 16S-23S rDNA ITS polymorphism analysis proved to be inadequate for species-level discrimination of psychrophilic and psychrotrophic clostridia associated with meat spoilage (Broda *et al*. 2003), and a drawback of the PCR-RFLP targeting *tpi* gene and automated ribotyping was that some species were difficult to definitively identify without supplementary testing (Dhalluin *et al*. 2003, Kennett and Stark 2006). The MicroSeq 500 16S rDNA bacterial identification system has proved to be better in identifying clostridia than commercial identification kits (Lau *et al*. 2006). However, the database needs to be expanded to also be able to identify rarely occurring clostridia (Woo *et al*. 2003, 2006, 2007, Lau *et al*. 2006). In addition, although 16S rRNA sequence analysis has proved to be suitable for differentiation of some *Clostridium* species and has successfully been applied to identify medically important clostridia (Lawson *et al*. 1993, Collins *et al*. 1994, Brazier *et al*. 2002, Woo *et al*. 2005, Decousser *et al*. 2007, Fujitani *et al*. 2007), certain species, such as *C. novyi* and *C. botulinum* type C, *C. ghoni* and *C. sordellii*, as well as proteolytic *C. botulinum* and *C. sporogenes* show very high 16S rRNA sequence similarity and may thus be difficult to differentiate using this method (Hutson *et al*. 1993, Lawson *et al*. 1993). *In silico* analysis of 16S rRNA gene sequencing-based methods has also shown that various methods were able to identify only 31-55% of the 42 *Clostridium* species included in the study. Among the *Clostridium* species that 16S rRNA sequences were unable to accurately speciate were the clinically important *C. botulinum*, *C. septicum*, *C. tertium* and *C. tetani* (Woo *et al*. 2007).

For common or clinically important clostridia, numerous identification techniques, such as species-specific PCR tests and techniques for rapid detection of species- or type-specific toxins, have been established (Hatheway 1990, Knoop *et al*. 1993, Brynestad and Granum 2002, Wilkins and Lyerly 2003, Heikinheimo and Korkeala 2005, Lindström and Korkeala 2006). However, the disadvantage of these techniques is that a specific test is required for each organism, and therefore, a wide selection of methods has to be set up to be able to identify several *Clostridium* species.

2.8.3 Clostridia in food

C. botulinum is divided into three groups based on phenotypic characteristics. Group I contains type A strains and proteolytic strains of type B and F, group II type E strains and non-proteolytic strains of type B and F, and group III type C and D strains (Lindström and Korkeala 2006). *C. botulinum* types A, B, E and F are mainly responsible for human botulism (Lynt *et al*. 1982, Hatheway 1995). Group I strains do not grow at temperatures below 10°C, but the spores have high heat resistance, and thus, group I strains may cause problems, especially in canning and home preservation of vegetables and meat. Group II strains, by contrast, are able to grow at 3°C, and their spores have lower heat resistance; these strains pose a safety risk particularly for refrigerated minimally processed packaged foods of extended durability (Lynt *et al*. 1982, Lindström *et al*. 2006). Foodborne botulism occurs after the ingestion of food containing preformed neurotoxin. Typical symptoms include nausea, vomiting, constipation and descending flaccid paralysis, finally resulting in failure of the respiratory musculature, which is why the disease is life-threatening if left untreated (Hatheway 1995). Often reported vehicles in botulism outbreaks are home-canned vegetables, fish or marine mammal products and different meat products (Roblot *et al*. 1994, Hatheway 1995, Boyer *et al*. 2001).

C. perfringens is divided into five types (A-E) based on the presence of genes encoding the four major lethal toxins (Petit *et al*. 1999). Enterotoxin-producing *C. perfringens* type A is widely recognized as one of most important causes of food poisoning of bacterial origin (Brynestad and Granum 2002, Lukinmaa *et al*. 2002, Lynch *et al*. 2006). The disease results from the ingestion of food containing large numbers of vegetative cells, which can sporulate and produce enterotoxin in the gastrointestinal tract (Rood and Cole 1991, Taormina and Dorsa 2004). Typical signs, *e.g.* diarrhoea, nausea and abdominal pain, last about 24 h, and due to the mildness of the disease, it is likely to be underreported (Hatheway 1990, Rood and Cole 1991, Brynestad and Granum 2002). Most *C. perfringens* outbreaks are caused by meat products, but fish and foods containing peas have also served as vehicles (Brunestad and Granum 2002, Taormina and Dorsa 2004, Lahti *et al*. 2008). Outbreaks typically occur in hospitals, restaurants or other food establishments where large amounts of food are prepared well in advance of service (Brynestad and Granum 2002). If cooling time of the food within the growth range of *C. perfringens* (15-50°C) is too slow and or the food is not sufficiently reheated, the number of organisms increase rapidly (Brynestad and Granum 2002, Taormina and Dorsa 2004).

Several *Clostridium* species are also challenging for the food processing industry due to their food spoilage properties. Various psychrophilic, psychrotrophic and mesophilic clostridia can cause deep tissue or “bone taint” spoilage of meat

(Boerema *et al*. 2002) or blown pack spoilage of vacuum-packed chilled meats (Collins *et al*. 1992, Broda *et al*. 1996, Boerema *et al*. 2003). In addition, *C. tyrobutyricum*, the causative agent of late blowing in cheese, causes considerable economic losses to cheese producers (Dasgupta and Hull 1989, Klijn *et al*. 1995).

3. AIMS OF THE STUDY

The objective of this work was to evaluate the applicability of AFLP in strain typing and identification of bacteria, with special attention directed to the foodborne pathogens *L. monocytogenes*, *C. botulinum* and *C. perfringens*. Specific aims were as follows:

1. to develop highly discriminative AFLP protocols for optimal characterization of *L. monocytogenes* and *C. botulinum* group I and II strains (I, II),

2. to assess the reproducibility, ease of performance, typeability and discriminatory power of the AFLP approach and to determine the suitability of AFLP analysis in typing *L. monocytogenes*, *C. botulinum* and *C. perfringens* at the strain level (I-V),

3. to evaluate the applicability of AFLP analysis in differentiation of *Listeria* and *Clostridium* species (I, III),

4. to investigate the contamination routes of *L. monocytogenes* using AFLP analysis in a chilled food processing plant producing ready-to-eat and ready-to-reheat foods and to evaluate the effect of different intervention methods on the occurrence of *L. monocytogenes* (IV) and

5. to examine the genetic similarity of persistent and sporadic *L. monocytogenes* strains using AFLP and PFGE analyses (V).

4. MATERIALS AND METHODS

4.1 Bacterial strains (I-III, V)

L. monocytogenes (*n*=89), *L. grayi* (*n*=1), *L. innocua* (*n*=3), *L. ivanovii* (*n*=2), *L. seeligeri* (*n*=2) and *L. welshimeri* (*n*=3) strains were selected from the culture collection of the Department of Food and Environmental Hygiene (I, V). In Study I, strains originated from various foods, animals, silage and the food processing environment. In addition, strains originating from the American Type Culture Collection (Manassas, VA, USA) and the National Collection of Type Cultures (London, UK) were included. In Study V, persistent (*n*=17) and sporadic (*n*=38) *L. monocytogenes* strains from 11 food processing plants were analysed. Strains were selected based on previous PFGE typing data and occurrence of pulsotypes in a plant. Strains were considered to be persistent if they were recovered recurrently from both the processing equipments and final products over a minimum of one year, whereas strains isolated sporadically from raw ingredients, environment or equipment but not from final products were considered to be sporadic.

A total of 173 strains of 24 different *Clostridium* species from the culture collection of the Department of Food and Environmental Hygiene, University of Helsinki, Finland; the Department of Medical Microbiology, University of Turku, Finland; the Institute of Food Research, Norwich, United Kingdom; and the Finnish Food Safety Authority, Kuopio Research Unit, Kuopio, Finland were analysed using AFLP (II, III). Strains consisted of type strains and strains originating from clinical, environmental and food samples.

4.2 Contamination route study (IV)

A total of 319 *L. monocytogenes* isolates were obtained as part of a quality control programme in a chilled food processing plant. Sampling was carried out during an eight-year period (May 1998-July 2006) and was especially targeted to post-heating areas, to equipment that is difficult to clean and to sites that had earlier tested positive for *L. monocytogenes*. In general, basic sampling was carried out on each line at least weekly. If *L. monocytogenes* was recovered, additional sampling was performed. Samples were collected both during production and after sanitization. Isolates originated from raw materials (*n*=18), equipment (*n*=193), the processing environment (*n*=77) and products (*n*=31). Sampling data were obtained from the records of the food processing plant.

The food processing plant used various raw ingredients, such as meat, fish, vegetables, dairy products and flour, in production of chilled ready-to-eat and ready-to-reheat foods as well as in foods requiring cooking before consumption. The plant was divided into three compartments with differing degrees of compartmentalization, and a total of eight processing lines (A-H) were used for production (Table 5). The cleaning services of the plant were outsourced and job rotation was utilized between departments; however, on one production day the assigned duties were only rotated within the department.

Table 5. Different compartments, production lines and product types of the plant.

Compartment[a]	Compartmentalization	Production lines	Products
I (1998-1999)	No compartmentalization	A, B, C, D, E	Cooked ready-to-eat and ready-to-reheat foods Uncooked products requiring cooking before consumption
I (2000-2006)	Raw and post-heat treatment areas separated	A, B, C, D, E	Cooked ready-to-eat and ready-to-reheat foods
II	No compartmentalization	F	Ready-to-eat and ready-to-reheat foods[b]
III	Raw and post-heat treatment areas separated	G, H	Cooked ready-to-reheat foods

[a] Sampling year in parentheses
[b] Products composed of cooked and uncooked ingredients. However, the finished products were not cooked in the plant.

4.3 AFLP analysis

4.3.1 DNA isolation of *Listeria* spp. (I, IV, V)

DNA was extracted according to the method of Pitcher *et al*. (1989), with slight modifications. Briefly, cells were harvested from a 1.5-ml volume of brain heart infusion (BHI) broth after incubation at 37°C for 14-16 h and resuspended with 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA) containing lysozyme 25 mg/ml (Sigma, St. Louis, MO, USA), mutanolysin 250 U/ml (Sigma) and RNase 100 µg/ml (Sigma) at

37°C for 1.5 h. The cells were lysed by addition of 500 µl of GES reagent (5 M guanidium thiocyanate, 100 mM EDTA, 0.5% [vol/vol] sarkosyl), cooled on ice for 5 min and then mixed with 250 µl of ammonium acetate (7.5 M). After incubation on ice for 10 min, chloroform-2-pentanol (24:1[vol/vol]) extraction was performed and DNA was precipitated with 2-propanol and washed with 75% (vol/vol) ethanol.

4.3.2 DNA isolation of *Clostridium* spp. (II, III)

In Study II, DNA was extracted according to the method of Hyytiä *et al*. (1999a). In Study III, the protocol was modified to enable completion of the extraction within one working day. Briefly, strains were cultivated in a trypticase-peptone-glucose-yeast medium (Lilly *et al*. 1971) under anaerobic conditions at the optimal growth temperature of each *Clostridium* species for 14-16 h. The cells were harvested from a 5-ml volume of overnight culture and lysed in TE (10 mM Tris-HCl, 1 mM EDTA) containing 7.9 mg/ml lysozyme (Sigma), 159 IU/ml mutanolysin (Sigma) and 467 µg/ml RNase (Sigma) at 37°C with gentle shaking for 15 min (*C. botulinum* group I), 2 h (*C. botulinum* group II) or 1 h (other clostridia). To obtain complete lysis, 52 µg/ml proteinase K (Finnzymes, Espoo, Finland), 0.23 M NaCl, 9.1 mM EDTA and 0.8% (vol/vol) sodium dodecyl sulphate were added, and, after thorough mixing, the mixture was incubated at 60 °C for 1 h with gentle shaking. Phenol-chloroform-isoamyl alcohol (25.24:1 [vol/vol]) and chloroform-2-pentanol (24:1 [vol/vol]) extractions were performed, and the DNA was ethanol (95% [vol/vol]) -precipitated and rinsed with 70% ethanol.

4.3.3 Determination of DNA concentrations (I-V)

DNA was resuspended with 100 µl of sterile, distilled, deionised water, and DNA concentrations were measured using a BioPhotometer (Eppendorf, Hamburg, Germany). DNA samples were stored at -70°C prior to AFLP analysis.

4.3.4 Initial testing (I, II)

During initial testing different restriction enzyme combinations and primer couplings were screened to find an enzyme-primer combination that generated evenly distributed banding patterns and also discriminated between closely related *L. monocytogenes* or *C. botulinum* strains (Table 6). For initial testing, strains of

L. monocytogenes (*n*=7) and strains of *C. botulinum* type A (*n*=3), B (*n*=2) and E (*n*=4) were selected based on previous PFGE typing data.

Table 6. Different restriction enzyme combinations and number of different primer combinations screened during initial testing.

Species	Restriction enzyme combination	No. of primer combinations
C. botulinum	*Apa*I - *Hpy*CH4IV	8
	*Eco*RI - *Hpy*CH4IV	2
	*Hin*dIII - *Hpy*CH4IV	12
	*Hin*dIII – *Mse*I	8
L. monocytogenes	*Apa*I - *Hpy*CH4IV	12
	*Apa*I – *Mse*I	12
	*Apa*I – *Taq*I	12
	*Eco*RI - *Hpy*CH4IV	12
	*Eco*RI – *Mse*I	12
	*Eco*RI – *Taq*I	12
	*Hin*dIII - *Hpy*CH4IV	12
	*Hin*dIII – *Mse*I	12
	*Hin*dIII – *Taq*I	12

4.3.5 AFLP reaction and electrophoresis (I-V)

The AFLP reactions were carried out essentially as described earlier (Vos *et al*. 1995, Thompson *et al*. 2001), with a few modifications. Total genomic DNA (400 ng) was digested with 15 U *Hin*dIII (New England Biolabs, Beverly, MA, USA) and 15 U *Hpy*CH4IV (New England Biolabs) in 1X One-Phor-All buffer plus (Amersham Biosciences, Buckinghamshire, UK), 5 MM dithiothreitol (DTT) and 0.1 mg/ml bovine serum albumin (BSA). Subsequently, restriction site-specific HindIII adapter (0.04 µM; Oligomer, Helsinki, Finland) and HpyCH4IV adapter (0.4 µM; Oligomer) (I, Table 3) were ligated with 1.1 U T4 DNA ligase (New England Biolabs) in 1 x One-Phor-All Buffer Plus (Amersham Biosciences), DTT 5 mM, BSA 0.1 mg/ml and ATP 200 µM. Samples were stored at -20°C prior to PCR amplification.

Restriction fragments with specific adapters were diluted with sterile, distilled, deionised water and amplified by preselective PCR (72ºC 2 min, 20 cycles [94ºC 20 s, 56ºC 2 min, 72ºC 2 min]) using primers without selective extension in a 20-µl reaction mixture containing 4 µl of diluted template DNA, 15 µl of Amplification Core Mix (Applied Biosystems, Foster City, CA, USA), 25 nM Hind-0

primer (Oligomer) and 125 nM Hpy-0 primer (Oligomer) (I, Table 3). Following preselective amplification, the samples were diluted with sterile, distilled, deionised water. Selective amplification was performed in a 10-µl reaction mixture containing 1.5 µl of diluted template, 50 nM Hind-A (I, IV, V) or Hind-C primer (II, III) (Oligomer), 250 nM Hpy-A primer (Oligomer) (I, Table 3) and 7.5 µl of Amplification Core Mix (94ºC 2 min, 1 cycle [94ºC 20 s, 66ºC 30 s, 72ºC 2 min]; then the annealing temperature was lowered by 1ºC in each cycle to 56ºC [10 cycles], followed by an additional 19 cycles at a 56ºC annealing temperature and a final 30-min extension at 60ºC). The selective Hind-A and Hind-C primers were either IRD800-labelled (I, II, V) or FAM-labelled (III, IV).

Denatured fragments were electrophoresed either on a 7% denaturing polyacrylamide gel in 1X Tris-borate-EDTA buffer on an automatic DNA sequencer (Li-COR Global IR2 4200LI-1 Sequencing system; LI-COR, Lincoln, NE, USA) (I, II, V) or on POP-4 polymer (Applied Biosystems) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) in 1 x Genetic Analyzer Buffer with EDTA (Applied Biosystems) (III, IV). IRDye800 50- to 700-bp sizing standard (LI-COR) (I, II, V) or GeneScan-500 LIZ size standard (Applied Biosystems) (III, IV) was used to enable fragment size determination.

4.3.6 Reproducibility testing (I-V)

Reproducibility of the method was determined by performing independent, repeated (duplicate, triplicate, fivefold or sixfold) experiments, including DNA extraction, AFLP analysis, electrophoresis and numerical data analysis, with seven *L. monocytogenes* strains (I), 38 *C. botulinum* strains (II) and 25 strains representing different *Clostridium* species (III). In addition, reproducibility among different data sets was assessed by using *L. monocytogenes* strain ATCC 15313 (I, IV, V) or *C. botulinum* strain K-51 (II, III) as an internal reference, which underwent each step of the DNA extraction and AFLP analysis, thereby providing a standard for comparison among different data sets.

4.4 In situ DNA isolation and PFGE (I, IV, V)

In situ DNA isolation and PFGE were performed as described by Autio *et al*. (2002). Briefly, strains were cultivated in BHI broth overnight at 37°C. The cells were harvested from a 2-ml volume of BHI broth in 5 ml of PIV (10 mM Tris [pH 7.5], 1 M NaCl), resuspended with PIV and mixed with an equal volume of 2% (wt/vol) low melting point agarose (InCert Agarose; FMC Bioproducts, Rockland, ME, USA).

GelSyringe dispensers (New England Biolabs) were used to form plugs. The plugs were incubated in lysis solution (6 mM Tris [pH 7.5], 1 M NaCl, 100 mM EDTA [pH 8.0], 0.5% Brij 58 [Sigma, St. Louis, MO, USA], 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 μg/ml RNase, 1 mg/ml lysozyme [Sigma] and 10 U/ml mutanolysin [Sigma]) at 37°C with gentle shaking for 3 h. This was followed by a 1-h wash with ESP (0.5 M EDTA [pH 8.0], 10% sodium lauroyl sarcosine, 100 μg/ml proteinase K [Finnzymes]) at 50°C. After proteolysis, the plugs were washed in TE for 1 h, and proteinase K was inactivated using 1 mM Pefablock SC (Roche Diagnostics, Mannheim, Germany) at 37°C overnight.

Agarose-embedded DNA was digested with *Asc*I (New England Biolabs) (I), or *Apa*I (New England Biolabs) and *Asc*I (IV). In Study V, at least two restriction enzymes, *Asc*I, and *Apa*I or *Sma*I, or both, were used in selection of persistent and sporadic strains. Digestions were performed according to the manufacturer's instructions.

DNA fragments were electrophoresed through 1.0% (wt/vol) agarose gel (SeaKem gold; FMC Bioproducts) in 0.5 X TBE buffer (45 mM Tris, 4.5 mM boric acid [pH 8.3] and 1 mM sodium EDTA) at 200 V at 14°C with the pulse times ramped from 1 s to 15 s for 18 h for *Sma*I and from 1 s to 35 s for 18 h for *Apa*I and *Asc*I using a Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala, Sweden). Low Range PFG marker (New England Biolabs) was used for fragment size determination. The gels were stained with ethidium bromide and digitally photographed under UV transillumination with an Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, CA, USA).

4.5 AFLP and PFGE pattern analyses (I-V)

The AFLP and PFGE patterns were analysed using BioNumerics software version 2.5, 3.0, 4.5 or 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities between normalized AFLP patterns were calculated with the Pearson product-moment correlation coefficient, and the similarity analysis of PFGE patterns was performed using the Dice coefficient (position tolerance 1%). Clustering and construction of dendrograms were performed by using the unweighted pair-group method with arithmetic averages.

4.6 Discrimination index (I, V)

The discriminatory power of AFLP and PFGE was compared by using Simpson's index of diversity (Hunter and Gaston 1988). The index estimates the probability that two unrelated strains sampled from a test population will be placed into different typing groups. The discrimination index (D) is given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

, where N is the total number of strains, s is the total number of types described and nj is the number of strains belonging to the jth type.

4.7 Serotyping of *L. monocytogenes* (I, IV)

Serotyping of *L. monocytogenes* strains was carried out using commercial Listeria antisera in accordance with the manufacturer's instructions (Denka Seiken, Tokyo, Japan). In Study I, all strains were serotyped, whereas in Study IV one to three strains from each AFLP type were randomly selected for serotyping.

4.8 PCR analysis of *C. botulinum* (II)

A previously described multiplex-PCR assay for the simultaneous detection of *C. botulinum* types A, B, E and F (Lindström *et al*. 2001) or PCR assay for separate detection of type A, B and E neurotoxin genes in *C. botulinum* (Franciosa *et al*. 1994) was applied to confirm the type of each *C. botulinum* strain.

4.9 Statistical analysis (IV)

Associations between AFLP types and compartments or lines were determined by categorical analysis with a Chi-square test or Fisher's exact test if expected values were less than five.

5. RESULTS

5.1 Suitable restriction enzyme and primer couplings for AFLP analysis (I, II)

Of the different enzyme combinations tested for AFLP analysis of *L. monocytogenes* and *C. botulinum*, couplings containing *Hin*dIII or *Eco*RI yielded a manageable number of bands and evenly distributed banding profiles. Combinations containing *Apa*I, by contrast, generated less than 20 fragments regardless of the primer coupling used and were thus deemed unsuitable for AFLP analysis. Based on amplification of relatively large numbers of evenly distributed DNA fragments, consistently strong signals on gels and detection of polymorphism among closely related strains (based on PFGE), selective primer combinations Hind-A and Hpy-A, and Hind-C and Hpy-A were selected for further analysis of *L. monocytogenes* (I) and *C. botulinum* (II), respectively.

5.2 Reproducibility and typeability of AFLP analysis (I-V)

All *Listeria* and *Clostridium* strains were typeable by AFLP, and hence, the typeability of the method was 100%. In reproducibility testing, the independent repeated experiments resulted in identical AFLP banding profiles (I-III). Furthermore, the internal reference strains *L. monocytogenes* ATCC 15313 (I, IV, V) and *C. botulinum* K-51 (II, III) showed identical AFLP banding patterns, measured based on fragment sizes, during each separate run. However, small differences were detected in lane and background intensities (I, II, V) or in peak heights (III, IV), and therefore, the similarity between reference strains varied in different studies, being at the lowest 89%. Based on the similarity of internal reference strains, a cut-off value for AFLP type definition was determined, *i.e.* strains showing higher similarity than reference strains were considered to be the same AFLP type (I-III, V). In Study IV, selection of the cut-off value was based on visual examination of the banding profiles of all strains investigated. Visual examination of the banding patterns revealed, however, minor fragment differences in strains of six different AFLP types designated as identical with the selected cut-off value (I, III, IV).

5.3 Characterization of *L. monocytogenes* by AFLP and PFGE (I, IV, V)

5.3.1 AFLP and PFGE analyses of *L. monocytogenes* strains (I, IV, V)

Both AFLP and PFGE analyses yielded three genomic groups of *L. monocytogenes* strains (I). Genomic group I consisted of serotypes 1/2a, 1/2c and 3a, while serotypes 1/2b, 4b, 4c and 7 formed genomic group II. Serotype 4a strain belonged to genomic group III. With AFLP, some level of serotype-related subclustering was also observed (I, IV). *L. monocytogenes* strains of genomic groups I and II could be differentiated from other *Listeria* species by the presence of an AFLP fragment of 206 bp (I). In addition, a 209-bp fragment could be identified for strains belonging to *L. monocytogenes* genomic groups II and III. Fragments of 149 and 296 bp were group-specific to strains of genomic group I. However, a 149-bp fragment was also detected in AFLP patterns of *L. welshimeri* and *L. ivanovii*. Fragments specific for persistent or sporadic strains of *L. monocytogenes* were not detected by either AFLP or PFGE (V).

5.3.2 Discriminatory power (I, V)

In Study I, *L. monocytogenes* strains were divided into 29 AFLP types and 29 pulsotypes, whereas in Study V the corresponding figures were 36 and 46 (Table 7). Both AFLP and PFGE were able to further separate types of strains formed by the other method. The highest discriminatory power was achieved by combining the results of AFLP and PFGE.

Table 7. Discriminatory power of AFLP, PFGE and their combination determined by using Simpson's index of diversity.

Study	No. of AFLP types	No. of pulsotypes	No. of genotypes[a]	Discriminatory power		
				AFLP	PFGE	Combination
I	29	29[b]	31	0.988	0.991	0.995
V	36	46[c]	48	0.982	0.993	0.994

[a] Genotypes obtained by combining AFLP and PFGE typing results.
[b] Pulsotype determination based on *Asc*I macrorestriction patterns (MRPs)
[c] Pulsotype determination based on *Asc*I, and *Apa*I and/or *Sma*I MRPs

5.3.3 Characterization of persistent and sporadic *L. monocytogenes* strains (V)

With AFLP and PFGE, *L. monocytogenes* strains were divided into two distinct clusters, both of which consisted of persistent and sporadic strains. By combining the typing results of AFLP and PFGE, a total of 48 different genotypes were observed (Table 8). Of these genotypes, 13 and 33 were specific for persistent and sporadic strains, respectively, while only two genotypes contained both persistent and sporadic strains. Clusters specific for persistent or sporadic strains were not observed.

Table 8. Number of different genotypes of persistent and sporadic *L. monocytogenes* strains obtained using PFGE, AFLP or their combination.

Typing method	Total no. of types	No. of types among persistent strains	No. of types among sporadic strains
PFGE	46	14	35
AFLP	36	14	28
Combination	48	15 (13)[a]	35 (33)[b]

[a] Number of types specific for persistent strains in parentheses.
[b] Number of types specific for sporadic strains in parentheses.

5.4 Characterization of *C. botulinum* and *C. perfringens* by AFLP (II, III)

AFLP analysis clearly differentiated between group I (proteolytic; *n*=33) and group II (non-proteolytic; *n*=37) *C. botulinum*. The group-specific clusters were linked together with a <10% similarity level. Group II was further separated into three clusters; two clusters consisted of *C. botulinum* type E strains, while *C. botulinum* type B and F strains formed the third cluster. Group-specific fragments of 129, 145 and 336 bp were identified for all *C. botulinum* group I strains, whereas fragments of 114 and 315 bp were specific to *C. botulinum* group II strains. However, no *C. botulinum* species- or type-specific fragments were observed.

AFLP analysis divided *C. perfringens* strains (*n*=37) into two subclusters; subcluster 1 consisted of strains of toxin types A, B, C, D and E, while subcluster 2 contained only strains of toxin type A. With a 93% cut-off value, a total of 29 different AFLP types were identified. AFLP analysis of isolates originating from the same food poisoning outbreak resulted in identical fingerprinting patterns.

5.5 Application of AFLP for species identification (I, III)

AFLP distinguished *L. monocytogenes* from other *Listeria* species (I); the only *L. grayi* strain included in the study occupied a separate position, and strains of other *Listeria* species were divided into species-specific clusters with less than 33% similarity between different species.

Numerical analysis of AFLP profiles of 24 different *Clostridium* species yielded 21 clusters at the 45% similarity level (III). Thirteen species were separated into single species-specific clusters, and eight strains, which were the only representatives of the particular species studied, occupied separate positions. In addition, *C. botulinum* strains were divided into seven distinct species-specific clusters. AFLP failed, however, to discriminate between *C. ramosum* and *C. limosum* at the 45% similarity level.

Group I (proteolytic) *C. botulinum* strains formed a single cluster, whereas group II (non-proteolytic) strains were separated into three clusters; one cluster consisted of strains of *C. botulinum* types B and F, while *C. botulinum* type E strains were divided into two clusters. *C. novyi* and group III *C. botulinum* types C and D clustered together with a similarity value of 22%. *C. novyi* and *C. botulinum* type D showed single species-specific clusters, whereas *C. botulinum* type C strains were divided into two clusters. Although AFLP analysis clearly differentiated between *C. botulinum* and *C. sporogenes*, these species were linked together at a similarity value of 22%.

5.6 *L. monocytogenes* contamination pattern in a food processing plant (IV)

Altogether 319 *L. monocytogenes* isolates were collected during the eight-year surveillance. The isolates were divided into four serotypes and 18 different AFLP types, five of which were persistent (Table 9). Isolates (n=177) of compartment I belonged to three persistent AFLP types. AFLP type A1 persisted throughout the eight-year surveillance and clearly predominated; 93% of the isolates of the compartment I were type A1. One of the AFLP types, A11, was specific for compartment I. Most contaminated lines were A and B, with 99 and 59 isolates recovered, respectively. AFLP type A1 was also significantly more common ($P < 0.05$, Fisher's exact test) in lines A and B than in lines C, D and E. The highest number of different AFLP types was detected in compartment II; the isolates (n=92) were divided into four persistent and five non-persistent AFLP types. All non-persistent and two persistent AFLP types were specific for compartment II. In

compartment III, only one *L. monocytogenes* isolate, belonging to AFLP type A1, was recovered.

Raw ingredients and finished products were found to be positive for *L. monocytogenes* 18 and 31 times, respectively. Isolates recovered from raw ingredients (grated cheese, sweet pepper, cooked chicken product) belonged to one persistent and seven non-persistent AFLP types, whereas three persistent and four non-persistent AFLP types were observed in finished products. All products containing *L. monocytogenes* were non-heat-treated and produced in compartment II.

In compartment II, AFLP type A18 was isolated from both raw ingredients (grated cheese) and finished products 13 times during a three-month period. After replacement of the cheese supplier, AFLP type A18 was detected only once in a batch of grated cheese. This *L. monocytogenes*-positive batch of cheese was not used for production.

Table 9. *Listeria monocytogenes* AFLP types and serotypes recovered from different sampling sites.

Sampling site	Total no. of isolates	AFLP type	No. of isolates	Serotype	Persistence
Compartment I	177	A1	165	1/2a	Persistent
		A11	4	1/2a	Persistent
		A14	8	1/2a	Persistent
Compartment II	92	A1	8	1/2a	Persistent
		A3	1	1/2a	Non-persistent
		A5	1	1/2c	Non-persistent
		A7	53	1/2a	Persistent
		A8	1	1/2a	Non-persistent
		A9	1	1/2a	Non-persistent
		A10	13	1/2a	Persistent
		A13	1	1/2a	Non-persistent
		A14	13	1/2a	Persistent
Compartment III	1	A1	1	1/2a	Persistent
Raw materials	18	A4	1	1/2c	Non-persistent
		A6	1	1/2a	Non-persistent
		A7	4	1/2a	Persistent
		A8	1	1/2a	Non-persistent
		A15	1	1/2a	Non-persistent
		A16	1	4b	Non-persistent
		A17	1	1/2b	Non-persistent
		A18	8	4b	Non-persistent
Products[a]	31	A2	1	1/2a	Non-persistent
		A7	19	1/2a	Persistent
		A10	1	1/2a	Persistent
		A12	1	1/2a	Non-persistent
		A13	1	1/2a	Non-persistent
		A14	2	1/2a	Persistent
		A18	6	4b	Non-persistent

[a] All products containing *L. monocytogenes* were produced in compartment II.

6. DISCUSSION

6.1 Typeability, reproducibility and ease of performance of AFLP analysis (I-V)

When a new typing system is evaluated, several criteria, such as typeability, ease of interpretation and performance, discriminatory power and reproducibility, must be assessed to determine the performance of the technique and its suitability for different kinds of research (Maslow *et al*. 1993, Power 1996). The AFLP approach presented here showed excellent performance in terms of typeability: no *Listeria* and *Clostridium* strains failed to generate AFLP profiles. This makes AFLP an attractive genotyping method, especially for clostridia, since some *Clostridium* strains are reported to produce extracellular DNases, which may hamper the use of DNA fingerprinting methods such as PFGE (Kristjánsson *et al*. 1994, Hielm *et al*. 1998a, Hyytiä *et al*. 1999b, Sperner *et al*. 1999b, Bidet *et al*. 2000, Stolle *et al*. 2001, Klaassen *et al*. 2002, Schalch *et al*. 2003). With AFLP, good-quality fingerprints were obtained for all *Clostridium* strains, and thus, this technique seemed to overcome the problem of DNA degradation. Since DNA degradation and untypeability by PFGE have also been reported for other genera, including *Escherichia* (Izumiya *et al*. 1997, Heir *et al*. 2000), *Mycobacterium* (Picardeau *et al*. 1997), *Pseudomonas* (Barth and Pitt 1995) and *Vibrio* (Marshall *et al*. 1999), AFLP may prove to be a more applicable fingerprinting method for these species as well. Although nucleases can be rapidly inactivated during DNA purification, many DNA isolation methods still degrade DNA to fragments of around 50 kbp due to the influence of mechanical shearing forces (Boom *et al*. 1990, Klaassen *et al*. 2002). However, this degradation is unlikely to affect the outcome of AFLP analysis since in AFLP the amplified and analysed fragments are of small size, 50-500 bp (Antonishyn *et al*. 2000, Klaassen *et al*. 2002).

In the present study, the independent repeated experiments proved that AFLP is a highly reproducible technique. Furthermore, the internal reference samples showed 89-95% similarity. This level of reproducibility is in accordance with previous studies (Janssen *et al*. 1997, Sloos *et al*. 1998, Duim *et al*. 1999, Huys *et al*. 2000, On and Harrington 2000, On *et al*. 2004, Kuehni-Boghenbor *et al*. 2006, Wieland *et al*. 2006, Keller *et al*. 2007). Variance in peak heights or fragment intensities is a recognized phenomenon in AFLP analysis (Duim *et al*. 1999, Antonishyn *et al*. 2000, Sims *et al*. 2002, On *et al*. 2004, Hong *et al*. 2005). The clustering level of internal reference strains was therefore used to assign different AFLP types. Although using threshold based on reproducibility analysis or on knowledge of outbreak strains to define the strain type is common (Duim *et al*. 1999,

Lindstedt *et al*. 2000c, Gzyl *et al*. 2005, Siemer *et al*. 2005, Torpdahl *et al*. 2005, Johnsen *et al*. 2006c, Kuehni-Boghenbor *et al*. 2006), it is also generally accepted that AFLP profiles with more than 90% identity indicate related isolates, and thus, this level of similarity has been used to assign AFLP type (D'Agata *et al*. 2001, Georgnaras *et al*. 2001, Willems *et al*. 2001, Amonsin *et al*. 2002, Schouls *et al*. 2003, Shaaly *et al*. 2005). When either of the above-mentioned approaches is used for AFLP type definition, it is also essential to visually check the quality of the AFLP profiles and confirm the results of pattern analysis, although the numerical analysis can be partly automated (On and Harrington 2000, Fry *et al*. 2002). In this study, visual examination of banding profiles revealed some minor fragment differences in isolates of the same AFLP type, and further analysis by PFGE subdivided isolates into distinct genotypes, thus stressing the importance of visual examination. Coenye *et al*. (1999b) have shown that visual examination of AFLP profiles is also required when AFLP is used for species identification to avoid misidentification of some isolates.

AFLP was found to be a relatively fast method; the AFLP analysis, including numerical data analysis, could be completed within two working days when initiated with pure DNA. The AFLP approach was also less labour-intensive than PFGE. Since AFLP is a PCR-based technique, which can also be partly automated, it provides high throughput and is especially suited for screening large number of isolates, *e.g.* in contamination route studies. However, an automated sequencer and computer software for pattern analysis are essential when the fAFLP technique is used, and therefore, the method may not be applicable in smaller diagnostic laboratories.

6.2 Discriminatory power of AFLP (I, V)

The discriminatory power of AFLP was compared with that of PFGE, which is considered to be the gold standard for molecular fingerprinting of many bacteria, including *L. monocytogenes* (Borucki *et al*. 2004). In Study I, both AFLP and PFGE were highly discriminatory, while in Study V the discriminatory power of PFGE was notably higher. However, in the latter study, the PFGE analysis was performed with two or three restriction enzymes to maximize the sensitivity of the method. Similarly, a combination of two or three different enzyme and primer couplings could also substantially add to the discriminatory power of AFLP and improve the value of this approach (Grady *et al*. 1999, Lan and Reeves 2002). High discriminative ability of AFLP for characterization of *L. monocytogenes* has also been reported by Fonnesbech Vogel *et al*. (2004), who found that AFLP showed higher discriminatory power than PFGE, RAPD and ribotyping.

Although the results of AFLP analysis were in agreement with those obtained by PFGE, some strains differentiated by AFLP were regarded as identical by PFGE, and vice versa. The highest discriminatory power was achieved by combining the results of AFLP and PFGE. This is in accordance with earlier studies, which have shown that it is essential to use a combination of different typing approaches when maximum type differentiation is needed, *e.g.* in outbreak investigations (Fonnesbech Vogel *et al*. 2004, Wittwer *et al*. 2005, Keller *et al*. 2007). In addition, it is necessary to know the overall genetic structure of the natural bacterial population and to take into account the epidemiological context to be able to interpret the typing results (Speijer *et al*. 1999, Goulding *et al*. 2000b, Grundmann *et al*. 2002). Smith *et al*. (2000) have even suggested that strains showing one- to two-fragment differences can be defined as either different strains or assigned to the same AFLP type; the decision should be determined in practice by the epidemiological context. It should also be borne in mind that each fingerprinting technique has both strengths and weaknesses and no single method is sufficient to comprehensively study the genetic relatedness among strains. The choice of methods depends on the research question, the genetic resolution needed, financial resources, available expertise and the technical facilities available (Mueller and Wolfenbarger 1999, Meays *et al*. 2004).

6.3 Characterization of *L. monocytogenes*, *C. botulinum* and *C. perfringens* strains by AFLP (I-V)

The results of the initial testing showed that enzyme combination *Hin*dIII and *Hpy*CH4IV and primer couplings Hind-A and Hpy-A, and Hind-C and Hpy-A for *L. monocytogenes* and *C. botulinum*, respectively, generated evenly distributed AFLP banding profiles and detected polymorphism among closely related strains. These combinations can therefore be recommended for AFLP analysis of *L. monocytogenes* and *C. botulinum*.

L. monocytogenes strains were divided into two or three different genogroups by both AFLP and PFGE. The AFLP results provide further evidence of the existence of three genetic lineages of *L. monocytogenes*. Three distinct lineages have been revealed by sequence analysis of listeriolysin O (*hly*), invasion-associated protein (*iap*) and flagellin (*flaA*) genes, ribotyping, virulence-associated gene polymorphism, PFGE and microarray data (Rasmussen *et al*. 1995, Wiedmann *et al*. 1997, Chasseignaux *et al*. 2001, Zhang *et al*. 2003, Sauders *et al*. 2006). In Study V, wide genetic diversity was observed among persistent and sporadic *L. monocytogenes* strains. The persistent strains differed from sporadic ones; the genotypes were mainly specific to either persistent or sporadic strains, and only two of the 48 genotypes

contained both persistent and sporadic strains. However, no specific clusters for persistent strains were observed, suggesting that no specific evolutionary lineage of persistent strains exists.

AFLP analysis clearly differentiated between group I (proteolytic) and group II (non-proteolytic) *C. botulinum* strains. Therefore, AFLP proved to be a suitable tool for *C. botulinum* group identification. This finding is in agreement with recent AFLP studies by Hill *et al*. (2007) and Macdonald *et al*. (2008). Although some type-specific subclustering was also observed, no type-specific fragments were detected, and thus, the AFLP approach was shown to be unsuitable for type determination of *C. botulinum* types A, B and F. However, type E-specific clusters suggest that AFLP has potential to define type E of *C. botulinum*. Similarly, Hill *et al*. (2007) showed that most type E and proteolytic type F strains formed type-specific clusters, whereas types A and B were not clearly differentiated by AFLP. Extensive genetic diversity detected among group II type E strains is in accordance with earlier studies conducted with PFGE (Hielm *et al*. 1998b, Hyytiä *et al*. 1999b). In addition, the observed high similarity of AFLP profiles of non-proteolytic type B and F strains is supported by a later AFLP study (Hill *et al*. 2007). In group I, less genetic variability was observed than in group II. However, AFLP was able to differentiate between group I *C. botulinum* and *C. sporogenes*, which is considered a non-toxigenic counterpart of group I *C. botulinum* and shows high 16S rRNA sequence homology and DNA relatedness with it (Lee and Rieman 1970, Hutson *et al*. 1993).

AFLP analysis of unrelated *C. perfringens* strains resulted in divergent fingerprints, whereas identical banding patterns were observed for strains initially originating from the same isolate or from the same outbreak, indicating that AFLP is a suitable tool for *C. perfringens* strain characterization. AFLP failed to differentiate between various toxinotypes of *C. perfringens*. However, this finding was expected since genes encoding three major toxins of *C. perfringens* (β, ε and ι) are located on plasmids, and loss or acquisition of a plasmid may even account for toxinotype change of a strain (Petit *et al*. 1999). Sawires and Songer (2006) reported the existence of strains of two different toxin types in the same clone by multiple-locus variable-number tandem repeat analysis, which suggests that the acquisition of plasmid-borne major toxin genes is a rather recent event and that *C. perfringens* strains of different toxin types may not have distinct evolutionary histories.

Although AFLP showed evident potential for strain typing of *C. botulinum* and *C. perfringens*, a drawback of the present study is that the discriminatory power of the AFLP approach was not compared with alternative genotyping procedures. Therefore, further research is needed to evaluate the discriminatory ability of AFLP.

6.4 Evaluation of the suitability of AFLP for species identification (I, III)

In Study I, AFLP distinguished all *Listeria* species tested. The less than 33% similarity between different species suggests that AFLP may also serve in *Listeria* species identification. This finding is in agreement with Fonnesbech Vogel *et al*. (2004), who reported that *Listeria* strains were grouped according to species in a study where 96 strains of *L. monocytogenes* and 9 strains representing six other *Listeria* species were analysed using AFLP.

When AFLP analysis was applied to 129 strains representing 24 different *Clostridium* species in Study III, AFLP distinguished all species, except *C. limosum* and *C. ramosum*, at the 45% similarity level. Similar cut-off levels for species identification have been used in studies of other bacterial species (Huys *et al*. 1996a, 1996b, Koeleman *et al*. 1998, Chang *et al*. 2005, Gzyl *et al*. 2005, Hong *et al*. 2005, Taponen *et al*. 2007). Strains of the species *C. botulinum* were divided into seven species-specific clusters, while other species were separated into single species-specific clusters or occupied separate positions. However, the groupings of *C. botulinum* strains using AFLP analysis are in agreement with the phylogenetic finding based on 16S rRNA sequencing of three distinct lineages of *C. botulinum* groups I, II and III (Hutson *et al*. 1993, Hill *et al*. 2007). The same three groups can also be recognized on the basis of phenotypic criteria (Cato *et al*. 1986), and, in general, these groups should be defined as separate species within the genus *Clostridium* (Hutson *et al*. 1993, Collins and East 1998, Hill *et al*. 2007).

The AFLP approach proved to be promising for identifying both *Listeria* and *Clostridium* species. AFLP is particularly advantageous since it simultaneously allows differentiation at the strain level (Duim *et al*. 2001). Furthermore, if species-specific AFLP fragments are observed, they may contain DNA sequences unique to one or a very limited number of species. Therefore, isolation and sequencing of such fragments may aid in development of species-specific diagnostic tools (Jackson *et al*. 1999). However, to obtain reliable species identification, establishing an expandable identification library with several AFLP profiles of well-defined strains for each species is necessary (Duim 2001, Chang *et al*. 2005). AFLP analysis of larger numbers of strains of *C. limosum* and *C. ramosum* may also facilitate differentiation between these species since clustering of a single AFLP profile of a species may be incorrect (Duim *et al*. 2001). Further AFLP analysis involving larger numbers of strains is therefore warranted to confirm the validity of the technique for genomic identification of *Listeria* and *Clostridium* species and to assess whether it is a useful tool in polyphasic taxonomic studies.

6.5 Diversity and persistence of *L. monocytogenes* in a chilled food processing plant analysed by AFLP (IV)

The three compartments of the chilled food processing plant producing ready-to-eat and ready-to-reheat foods showed markedly different contamination statuses. Several factors either predisposing compartments or lines to persistent contamination or protecting them from contamination were identified. In compartment III, strict separation of raw and post-heat treatment areas seemed to protect processing lines from contamination. In compartment I, by contrast, initial contamination by persistent AFLP type A1 may have been introduced to the heavily contaminated compartment already in 1998, when an uncooked product was manufactured in line B of the compartment. This is in accordance with a previous study, which showed that poor compartmentalization both increased and prolonged contamination in a meat processing plant (Lundén *et al*. 2003b). Although raw and post-heat treatment areas of compartment I were separated in 2000, the existing *L. monocytogenes* contamination could not be eliminated. In compartment III, the only positive sample was detected on a movable container that had been transferred to the compartment without cleaning. Thus, to avoid spread of contamination within a plant, it is also necessary to limit the movement of personnel and equipment.

The cooking step seemed to limit the flow of sporadic strains into compartments I and III, which produced cooked meals. However, a high level of persistent contamination was observed, especially in two lines of compartment I. A product type that made mechanical cleaning of the two lines difficult and insufficient cleaning routines, such as cleaning of the lines only every other day during the high season, seemed to predispose lines A and B to persistent contamination. The three most contaminated lines (A, B and F) harboured *L. monocytogenes*, especially in coolers, conveyors and packing machines. This is in agreement with earlier studies, which have shown that complex processing lines are more prone to persistent contamination and machines with complex structure that hinders efficient cleaning favour the persistence of *L. monocytogenes* (Autio *et al*. 1999, Johansson *et al*. 1999, Miettinen *et al*. 1999a, Lyytikäinen *et al*. 2000, Dauphin *et al*. 2001, Fonnesbech Vogel *et al*. 2001, Lundén *et al*. 2002, 2003b, Thévenot *et al*. 2005, 2006b, Gudmundsdóttir *et al*. 2006, Nakamura *et al*. 2006, De Cesare *et al*. 2007). Comprehensive cleaning and disinfection practices have been shown to significantly reduce the level of *L. monocytogenes* contamination (Autio *et al*. 1999, Miettinen *et al*. 1999a, Lundén *et al*. 2002, Gudmundsdóttir *et al*. 2006, Nakamura *et al*. 2006). The present study also revealed that extensive reconstruction of the processing line in compartment II both reduced prevalence rates of *L. monocytogenes* and eliminated two persistent AFLP types.

In compartment III, the use of raw ingredients that rarely contain *L. monocytogenes* likely protected the processing lines from contamination. However, the high number of different AFLP types, both persistent and non-persistent, detected in compartment II may result from the range of different raw materials, such as vegetables, meat products and dairy products, used in preparation of meals. These ingredients might harbour *L. monocytogenes* (Farber and Peterkin 1991, Rudolf and Scherer 2001, Aguado *et al*. 2004, Thévenot *et al*. 2006b), thus introducing the organism to the processing environment and since the process did not include a lethal kill step for *L. monocytogenes* the organism could even be detected in finished products. In this study, this kind of contamination of finished products was shown to be caused by grated cheese, stressing that more attention should be paid to microbial quality control of raw ingredients. In addition, if high-risk raw materials are identified they should be heat-treated before use in production.

The present study showed that *L. monocytogenes* is able to persist for extended periods of time in a plant producing chilled ready-to-eat and ready-to-reheat meals. However, since ready meals contain multiple raw ingredients and each product has a distinct preparation process, the contamination level may vary significantly within a plant. Long-lasting surveillance and the use of efficient genotyping methods, such as AFLP, are among the key elements in tracing the sources of contamination, thus enabling production of *L. monocytogenes*-free ready meals.

7. CONCLUSIONS

1. The AFLP technique was tailored for optimal characterization of *L. monocytogenes* and *C. botulinum* strains. The enzyme coupling *Hin*dIII – *Hpy*CH4IV proved to be suitable for both *L. monocytogenes* and *C. botulinum* strains. Primer combinations Hind-A and Hpy-A, and Hind-C and Hpy-A for *L. monocytogenes* and *C. botulinum*, respectively, generated uniform distribution of DNA fragments and detected polymorphism among closely related strains. These combinations are therefore recommended for AFLP analysis of *L. monocytogenes* and *C. botulinum*.

2. AFLP analysis proved to be a highly reproducible, easy to perform and relatively fast method with high throughput. Furthermore, all *Listeria* and *Clostridium* strains were typeable by AFLP. AFLP showed potential to subtype *L. monocytogenes*, *C. botulinum* and *C. perfringens* strains and proved to be suitable also for *C. botulinum* group identification. In addition, when AFLP was applied to *L. monocytogenes* strains, its discriminatory power was shown to equal that of PFGE, which is considered the current gold standard for molecular fingerprinting of *L. monocytogenes*. These features make AFLP analysis a useful alternative to other genotyping methods in, for example, outbreak investigations and contamination route studies. Due to the high throughput of samples, the AFLP approach is especially suited for screening large numbers of isolates.

3. AFLP differentiated all *Listeria* and *Clostridium* species tested, except for *C. ramosum* and *C. limosum*, and thus, AFLP analysis was shown to be a promising tool for genomic identification of *Listeria* and *Clostridium*. AFLP may be used as an additional tool in species identification if an expandable identification library with several AFLP profiles of well-defined strains for each species is established.

4. The three compartments of a chilled food processing plant showed markedly different contamination statuses. In processing lines of cooked meals, insufficient cleaning routines, product types hampering the mechanical cleaning of the production line and lack of proper compartmentalization predisposed production lines to persistent contamination. Uncooked products were also contaminated via raw materials; special attention should therefore be paid to continuous quality control of raw ingredients when uncooked ready-to-eat foods are produced. Reconstruction of a production line both reduced prevalence rates of

L. monocytogenes and eliminated two persistent AFLP types. Hence, structural adjustments may facilitate the eradication of *L. monocytogenes* from the food processing environment.

5. *L. monocytogenes* strains causing persistent contamination in food processing plants were shown to differ from sporadic strains. However, no specific evolutionary lineage of persistent *L. monocytogenes* strains was observed.

8. REFERENCES

Aarts, H.J.M., van Lith, L.A.J.T., and Keijer, J. 1998. High-resolution genotyping of *Salmonella* strains by AFLP fingerprinting. Lett. Appl. Microbiol. 26:131-135.

Aarts, H.J.M., Hakemulder, L.E., and Van Hoef, A.M.A. 1999. Genomic typing of *Listeria monocytogenes* strains by automated laser fluorescence analysis of amplified fragment length polymorphism fingerprint patterns. Int. J. Food Microbiol. 49:95-102.

Abdulla, A., and Yee, L. 2000. The clinical spectrum of *Clostridium sordellii* bacteraemia: two case reports and a review of the literature. J. Clin. Pathol. 53:709-712.

Aguado, V., Vitas, A.I., and García-Jalón, I. 2004. Characterization of *Listeria monocytogenes* and *Listeria innocua* from vegetable processing plant by RAPD and REA. Int. J. Food Microbiol. 90:341-347.

Ahmed, N., Alam, M., Majeed, A.A., Rahman, S.A., Cataldi, A., Cousins, D., and Hasnain, S.E. 2003. Genome sequence based, comparative analysis of the fluorescent amplified fragment length polymorphism (FAFLP) of tubercle bacilli form seals provides molecular evidence for a new species within the *Mycobacterium tuberculosis* complex. Infect. Genet. Evol. 2:193-199.

Akiba, M., Uchida, I., Nishimori, K., Tanaka, K., Anzai, T., Kuwamoto, Y., Wada, R., Ohya, T., and Ito, H. 2003. Comparison of *Salmonella enterica* serovar Abortusequi isolates of equine origin by pulsed-field gel electrophoresis and fluorescent amplified-fragment length polymorphism fingerprinting. Vet. Microbiol. 92:379-388.

Alexander, C.J., Citron, D.M., Brazier, J.S., and Goldstein, E.J.C. 1995. Identification and antimicrobial resistance patterns of clinical isolates of *Clostridium clostridioforme*, *Clostridium innocuum*, and *Clostridium ramosum* compared with those of clinical isolates of *Clostridium perfringens*. J. Clin. Microbiol. 33:3209-3215.

Amonsin, A., Wellehan, J.F.X., Ling-Ling, L., Laber, J., and Kapur, V. 2002. DNA fingerprinting of *Pasteurella multocida* recovered from avian sources. J. Clin. Microbiol. 40: 3025-3031.

Antonishyn, N.A., McDonald, R.R., Chan, E.L., Horsman, G., Woodmansee, C.E., Falk, P.S., and Mayhall, C.G. 2000. Evaluation of fluorescence-based amplified fragment length polymorphism analysis for molecular typing in hospital epidemiology: comparison with pulsed-field gel electrophoresis for typing strains of vancomycin-resistant *Enterococcus faecium*. J. Clin Microbiol. 38: 4058-4065.

Arnold, C., Metherell, L., Clewley, J.P., and Stanley, J. 1999a. Predictive modelling of fluorescent AFLP: a new approach to the molecular epidemiology of *E. coli*. Res. Microbiol. 150:33-44.

Arnold, C., Metherell, L., Willshaw, G., Maggs, A., and Stanley, J. 1999b. Predictive fluorescent amplified-fragment length polymorphism analysis of *Escherichia coli*: high-resolution typing method with phylogenetic significance. J. Clin. Microbiol. 37: 1274-1279.

Aureli, P., Fiorucci, G.C., Caroli, D., Marchiaro, G., Novara, O., Leone, L., and Salmaso, S. 2000. An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. N. Engl. J. Med. 342:1236-1241.

Autio, T., Hielm, S., Miettinen, M., Sjöberg, A.-M., Aarnisalo, K., Björkroth, J., Mattila-Sandholm, T., and Korkeala, H. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. Appl. Environ. Microbiol. 65:150-155.

Autio, T., Säteri, T., Fredriksson-Ahomaa, M., Rahkio, M., Lundén, J., and Korkeala, H. 2000. *Listeria monocytogenes* contamination pattern in pig slaughterhouses. J. Food Prot. 63:1438-1442.

Autio, T., Lundén, J., Fredriksson-Ahomaa, M., Björkroth, J., Sjöberg, A.-M., and Korkeala, H. 2002. Similar *Listeria monocytogenes* pulsotypes detected in several foods originating from different sources. Int. J. Food Microbiol. 77:83-90.

Avrova, A.O., Hyman, L.J., Toth, R.L., and Toth, I.K. 2002. Application of amplified fragment length polymorphism fingerprinting for taxonomy and identification of the soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. Appl. Environ. Microbiol. 68:1499-1508.

Ball, L.M., Bes, M.A., Theelen, B., Boekhout, T., Egeler, R.M., and Kuijper, E.J., 2004. Significance of amplified fragment length polymorphism in identification and epidemiological examination of *Candida* species colonization in children undergoing allogeneic stem cell transplantation. J. Clin. Microbiol. 41:1673-1679.

Barash, J.R., Tang, T.W.H., and Arnon, S.S. 2005. First case of infant botulism caused by *Clostridium baratii* type F in California. J. Clin. Microbiol. 43:4280-4282.

Barth, A.L., and Pitt, T.L. 1995. Auxotrophic variants of *Pseudomonas aeruginosa* are selected from prototrophic wild-type strains in respiratory infections in patients with cystic fibrosis. J. Clin. Microbiol. 33:37-40.

Benediktsdóttir, E., Verdonck, L., Spröer, C., Helgason, S., and Swings, J. 2000. Characterization of *Vibrio viscosus* and *Vibrio wodanis* isolated at different geographical locations: a proposal for reclassification of *Vibrio viscosus* as *Moritella viscosa* comb. nov. Int. J. Syst. Evol. Micr. 50:479-488.

Bensch, S., and Åkesson, M. 2005. Ten years of AFLP in ecology and evolution: why so few animals? Mol. Ecol. 14:2899-2914.

Berrang, M.E., Meinersmann, R.J., Northcutt, J.K., and Smith, D.P. 2002. Molecular characterization of *Listeria monocytogenes* isolated from a poultry further processing facility and from fully cooked product. J. Food. Prot. 65:1574-1579.

Bērziņš, A., Hörman, A., Lundén, J., and Korkeala, H. 2007. Factors associated with *Listeria monocytogenes* contamination of cold-smoked pork products produced in Latvia and Lithuania. Int. J. Food Microbiol. 115:173-179.

Bidet, P., Lalande, V., Salauze, B., Burghoffer, B., Avesani, V., Delmée, M., Rossier, A., Barbut, F., and Petit J.C. 2000. Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. J. Clin. Microbiol. 38:2484-2487.

Bikandi, J., Millán, R.S., Rementeria, A., and Garaizar, J. 2004. *In silico* analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. Bioinformatics 20:798-799.

Bitti, A., Mastrantonio, P., Spigaglia, P., Urru, G., Spano, A.I., Moretti, G., and Cherchi, G.B. 1997. A fatal postpartum *Clostridium sordellii* associated toxic shock syndrome. J. Clin. Pathol. 50:259-260.

Blears, M.J., De Grandis, S.A., Lee, H., and Trevors, J.T. 1998. Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. J. Ind. Microbiol. Biotechnol. 21:99-114.

Blears, M.J., Pokorny, N.J., Carreno, R.A., Chen, S., De Grandis, S.A., Lee, H., and Trevors, J.T. 2000. DNA fingerprinting of *Cryptosporidium parvum* isolates using amplified fragment length polymorphism (AFLP). J. Parasitol. 86:838-841.

Boerema, J.A., Broda, D.M., and Bell, R.G. 2002. PCR detection of psychrotolerant clostridia associated with deep tissue spoilage of vacuum-packed chilled meats. Lett. Appl. Microbiol. 35:446-450.

Boerema, J.A., Broda, D.M., and Bell, R.G. 2003. Abattoir sources of psychrophilic clostridia causing blown pack spoilage of vacuum-packed chilled meats determined by culture-based and molecular detection procedures. Lett. Appl. Microbiol. 36:406-411.

Boerema, J.A., Clemens, R., and Brightwell, G. 2006. Evaluation of molecular methods to determine enterotoxigenic status and molecular genotype of bovine, ovine, human and food isolates of *Staphylococcus aureus*. Int. J. Food Microbiol. 107:192-201.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., and van der Noordaa, J. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28:495-503.

Bootsma, H.J., van der Heide, H.G.J., van de Pas, S., Schouls, L.M., and Mooi, F.R. 2000. Analysis of *Moraxella catarrhalis* by DNA typing: evidence for a distinct subpopulation associated with virulence traits. J. Infect. Dis. 181:1376-1387.

Borriello, S.P., and Carman, R.J. 1983. Association of iota-like toxin and *Clostridium spiroforme* with both spontaneous and antibiotic-associated diarrhea and colitis in rabbits. J. Clin. Microbiol. 17:414-418.

Borst, A., Theelen, B., Reinders, E., Boekhout, T., Fluit, A.C., and Savelkoul, P.H.M. 2003. Use of amplified fragment length polymorphism analysis to identify medically important *Candida* spp., including *C. dubliniensis*. J. Clin. Microbiol. 41:1357-1362.

Borucki, M.K., Krug, M.J., Muraoka, W.T., and Call, D.R. 2003a. Discrimination among *Listeria monocytogenes* isolates using a mixed genome DNA microarray. Vet. Microbiol. 92:351-362.

Borucki, M.K., Peppin, J.D., White, D., Loge, F., and Call, D.R. 2003b. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. 69:7336-7342.

Borucki, M.K., Kim, S.H., Call, D.R., Smole, S.C., and Pagotto, F. 2004. Selective discrimination of *Listeria monocytogenes* epidemic strains by a mixed-genome DNA microarray compared to discrimination by pulsed-field gel electrophoresis, ribotyping, and multilocus sequence typing. J. Clin. Microbiol. 42:5270-5276.

Boumedine, K.S., and Rodolakis, A. 1998. AFLP allows the identification of genomic markers of ruminant *Chlamydia psittaci* strains useful for typing and epidemiological studies. Res. Microbiol. 149:735-744.

Boyer, A., Girault, C., Bauer, F., Korach, J.-M., Salomon, J., Moirot, E., Leroy, J., and Bonmarchand, G. 2001. Two cases of foodborne botulism type E and review of epidemiology in France. Eur. J. Clin. Microbiol. Infect. Dis. 20:192-195.

Brazier, J.S., Duerden, B.I., Hall, V., Salmon, J.E., Hood, J., Brett, M.M., McLauchlin, J., and George, R.C. 2002. Isolation and identification of *Clostridium* spp. from infections associated with the injection of drugs: experiences of a microbiological investigation team. J. Med. Microbiol. 51:985-989.

Brazier, J.S., Gal, M., Hall, V., and Morris, T.E. 2004. Outbreak of *Clostridium histolyticum* infections in injecting drug users in England and Scotland. Euro Surveill. 9:15-16.

Brett, M.M., McLauchlin, J., Harris, A., O'Brien, S., Black, N., Forsyth, R.J., Roberts, D., and Bolton, F.J. 2005. A case of infant botulism with a possible link to infant formula milk powder: evidence for the presence of more than one strain of *Clostridium botulinum* in clinical specimens and food. J. Med Microbiol. 54:769-776.

Brett, M.S.Y., Short, P., and McLauchlin, J. 1998. A small outbreak of listeriosis associated with smoked mussels. Int. J. Food Microbiol. 43:223-229.

Breyne, P., Dreesen, R., Cannoot, B., Rombaut, D., Vandepoele, K., Rombauts, S., Vanderhaeghen, R., Inzé, D., and Zabeau, M. 2003. Quantitative cDNA-AFLP analysis for genome-wide expression studies. Mol. Gen. Genomics 269:173-179.

Briard, M., Le Clerc, V., Grzebelus, D., Senalik, D., and Simon, P.W. 2000. Modified protocols for rapid carrot genomic DNA extraction and AFLP analysis using silver stain and radioisotopes. Plant Mol. Biol. Rep. 18:235-241.

Brisse, S., Fussing, V., Ridwan, B., Verhoef, J., and Willems, R.J.L. 2002. Automated ribotyping of vancomycin-resistant *Enterococcus faecium* isolates. J. Clin. Microbiol. 40:1977-1984.

Broda, D.M., DeLacy, K.M., Bell, R.G., Braggins, T.J., and Cook, R.L. 1996. Psychrotrophic *Clostridium* spp. Associated with "blown pack" spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int. J. Food Microbiol. 29:335-352.

Broda, D.M., Musgrave, D.R., and Bell, R.G. 2003. Molecular differentiation of clostridia associated with "blown pack" spoilage of vacuum-packed meats using internal transcribed spacer polymorphism analysis. Int. J. Food Microbiol. 84:71-77.

Brynestad, S., and Granum, P.E. 2002. *Clostridium perfringens* and foodborne infections. Int. J. Food Microbiol. 74:195-202.

Burlage, R.S., and Ellner, P.D. 1985. Comparison of the PRAS II, AN-Ident, and RapID-ANA systems for identification of anaerobic bacteria. J. Clin. Microbiol. 22:32-35.

Burtscher, M.M., Köllner, K.E., Sommer, R., Keiblinger, K., Farnleitner, A.H., and Mach, R.L. 2006. Development of a novel amplified fragment length polymorphism (AFLP) typing method for enterococci isolates from cattle faeces and evaluation of the single versus pooled faecal sampling approach. J. Microbiol. Meth. 67:281-293.

Carlier, J.-P., K'ouas, G., Lozniewski, A., Sirveaux, F., Caillous, P., and Mory, F. 2004. Osteosynthesis-associated bone infection caused by a nonproteolytic, nontoxigenic *Clostridium botulinum*-like strain. J. Clin. Microbiol. 42:484-486.

Carlier, J.-P., Manich, M., Loïez, C., Migaud, H., and Courcol, R.J. 2006. First isolation of *Clostridium amygdalinum* from a patient with chronic osteitis. J. Clin. Microbiol. 44:3842-3844.

Cato, E.P., George, W., and Finegold, S.M. 1986. Genus *Clostridium*. In Bergeys Manual of Systematic Bacteriology, Vol. 2. Sneath, P.H.A., Mair, N.S., and Sharpe, M.E. *et al*. (eds). Williams & Wilkins, Baltimore, pp. 1141-1200.

Celig, D.M., and Schreckenberger, P.C. 1991. Clinical evaluation of the rapid-ANA II panel for identification of anaerobic bacteria. J. Clin. Microbiol. 29:457-462.

Champion, O.L., Best, E.L., and Frost, J.A. 2002. Comparison of pulsed-field gel electrophoresis and amplified fragment length polymorphism techniques for investigating outbreaks of enteritis due to campylobacters. J. Clin. Microbiol. 40:2263-2265.

Chang, H.C., Wei, Y.F., Dijkshoorn, L., Vaneechoutte, M., Tang, C.T., and Chang, T.C. 2005. Species-level identification of isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex by sequence analysis of the 16S-23S rRNA gene spacer region. J. Clin. Microbiol. 43:1632-1639.

Chasseignaux, E., Toquin, M.-T., Ragimbeau, C., Salvat, G., Colin, P., and Ermel, G. 2001. Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry- and pork-processing plants. J. Appl. Microbiol. 91:888-899.

Clarridge III, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17:840-862.

Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J.R.W., Kersters, K., and Vandamme, P. 1999a. Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. Int. J. Syst. Bacteriol. 49:405-413.

Coenye, T., Schouls, L.M., Ovan, J.R.W., Kersters, K., and Vandamme, P. 1999b. Identification of *Burkholderia* species and genomovars from cystic fibrosis patients by AFLP fingerprinting. Int. J. Syst. Bacteriol. 49:1657-1666.

Coenye, T, LiPuma, J.J., Henry, D., Hoste, B., Vandemeulebroecke, K., Gillis, M., Speert, D.P., and Vandamme, P. 2001. *Burkhoderia cepacia* genomovar VI, a new member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. Int. J. Syst. Evol. Micr. 51:271-279.

Collins, M.D., Rodrigues, U.M., Dainty, R.H., Edwards, R.A., and Roberts, T.A. 1992. Taxonomic studies on a psychrophilic *Clostridium* from vacuum-packed beef: description of *Clostridium estertheticum* sp. nov. FEMS Microbiol. Lett. 96:235-240.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H., and Farrow, J.A.E. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44:812-826.

Collins, M.D., and East, A.K. 1998. Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. J. Appl. Microbiol. 84:5-17.

Coque, T.M., Willems, R.J.L., Fortún, J., Top, J., Diz, S., Loza, E., Cantón, R., and Baquero, F. 2005. Population structure of *Enterococcus faecium* causing bacteremia in a Spanish university hospital: setting the scene for a future increase in vancomycin resistance? Antimicrob. Agents Ch. 49:2693-2700.

Corcoran, D., Clancy, D., O´Mahony, M., Grant, K., Hyland, E., Shanaghy, N., Whyte, P., McLauchlin, J., Moloney, A., and Fanning, S. 2006. Comparison of *Listeria monocytogenes* strain types in Irish smoked salmon and other foods. Int. J. Hyg. Environ. Health 209:527-534.

D'Agata, E.M.C., Gerrits, M.M., Tang, Y.-W., Samore, M., and Kusters, J.G. Comparison of pulsed-field gel electrophoresis and amplified fragment-length polymorphism for epidemiological investigations of common nosocomial pathogens. Infect. Control Hosp. Epidemiol. 22:550-554.

Dalton, C.B., Austin, C.C., Sobel, J., Hayes, P.S., Bibb, W.F., Graves, L.M., Swaminathan, B., Proctor, M.E., and Griffin, P.M. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. N. Engl. J. Med. 336:100-105.

Dasgupta, A.P., and Hull, R.R. 1989. Late blowing of Swiss cheese: incidence of *Clostridium tyrobutyricum* in manufacturing milk. Aust. J. Dairy Technol. 44:82-87.

Dauphin, G., Ragimbeau, C., and Malle, P. 2001. Use of PFGE typing for tracing contamination with *Listeria monocytogenes* in three cold-smoked salmon processing plants. Int. J. Food Microbiol. 64:51-61.

De Boer, P., Duim, B., Rigter, A., van der Plas, J., Jacobs-Reitsma, W.F., and Wagenaar, J.A. 2000. Computer-assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 38:1940-1946.

De Cesare, A., Manfreda, G., Macrì, M., and Cantoni, C. 2007. Application of automated ribotyping to support the evaluation of *Listeria monocytogenes* sources in Taleggio cheese producing plant. J. Food. Prot. 70:1116-1121.

De Valk, H., Vaillant, V., Jacquet, C., Rocourt, J., Le Querrec, F., Stainer, F., Quelquejeu, N., Pierre, O., Pierre, V., Desenclos, J.-C., and Goulet, V. 2001. Two consecutive nationwide outbreaks of listeriosis in France, October 1999-February 2000. Am. J. Epidemiol. 154:944-950.

De Valk, H.A., Meis, J.F.G.M., de Pauw, B.E., Donnelly, P.J., and Klaassen, C.H.W. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J. Clin Microbiol. 45: 1415-1419.

De Zoysa, A., and Efstratiou, A. 2000. Use of amplified fragment length polymorphism for typing *Corynebacterium diphteriae*. J. Clin. Microbiol. 38:3843-3845.

Decousser, J.W., Bartizel, C., Zamni, M., Fadel, N., and Doucet-Populaire, F. 2007. *Clostridium symbiosum* as a cause of bloodstream infection in an immunocompetent patient. Anaerobe 13:166-169.

Dellagi, A., Birch, P.R.J., Heilbronn, J., Lyon, G.D., and Toth, I.K. 2000. cDNA-AFLP analysis of differential gene expression in the prokaryotic plant pathogen *Erwinia carotovora*. Microbiology 146:165-171.

Desai, M., Tanna, A., Wall, R., Efstratiou, A., George, R., and Stanley, J. 1998. Fluorescent amplified-fragment length polymorphism analysis of an outbreak of group A streptococcal invasive disease. J. Clin. Microbiol. 36: 3133-3137.

Desai, M., Logan, J.M.J., Frost, J.A., and Stanley, J. 2001a. Genome sequence-based fluorescent amplified fragment length polymorphism of *Campylobacter jejuni*, its relationship to serotyping, and its implications for epidemiological analysis. J. Clin. Microbiol. 39:3823-3829.

Desai, M., Threlfall, E.J., and Stanley, J. 2001b. Fluorescent amplified-fragment length polymorphism subtyping of the *Salmonella enterica* serovar Enteritidis phage type 4 clone complex. J. Clin. Microbiol. 39:201-206.

Destro, M.T., Leitão, M.F.F., and Farber, J.M. 1996. Use of molecular typing methods to trace the dissemination of *Listeria monocytogenes* in a shrimp processing plant. Appl. Environ. Microbiol. 62:705-711.

Dhalluin, A., Lemée, L., Pestel-Caron, M., Mory, F., Leluan, G., Lemeland, J.-F., and Pons, J.-L. 2003. Genotypic differentiation of twelve *Clostridium* species by polymorphism analysis of the triosephosphate isomerase (*tpi*) gene. System. Appl. Microbiol. 26:90-96.

Dice, L.R. 1945. Measures of the amount of ecologic association between species. Ecology 26:297-302.

Duim, B., Wassenaar, T.M., Rigter, A., and Wagenaar, J. 1999. High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. Appl. Environ. Microbiol. 65:2369-2375.

Duim, B., Ang, C.W., van Belkum, A., Rigter, A., van Leeuwen, N.W.J., Endtz, H.P., and Wagenaar, J.A. 2000. Amplified fragment length polymorphism analysis of *Campylobacter jejuni* strains isolated from chickens and from patients with gastroenteritis or Guillain-Barré or Miller Fisher syndrome. Appl. Environ. Microbiol. 66:3917-3923.

Duim, B., Vandamme, P.A.R., Rigter, A., Laevens, S., Dijkstra, J.R., and Wagenaar, J.A. 2001. Differentiation of *Campylobacter* species by AFLP fingerprinting. Microbiology 147:2729-2737.

Duim, B., Wagenaar, J.A., Dijkstra, J.R., Goris, J., Endtz, H.P., and Vandamme, P.A.R. 2004. Identification of distinct *Campylobacter lari* genogroups by amplified fragment length polymorphism and protein electrophoretic profiles. Appl. Environ. Microbiol. 70:18-24.

Elsayed, S., and Zhang, K. 2004. Human infection caused by *Clostridium hathewayi*. Emerg. Infect. Dis. 10:1950-1952.

Elsayed, S., and Zhang, K. 2007. *Clostridium glycolicum* bacteremia in a bone marrow transplant patient. J. Clin. Microbiol. 45:1652-1654.

Euzéby, J.P. 1997. List of bacterial names with standing in nomenclature: a folder available on the internet. (List of prokaryotic names with standing in nomenclature. Last full update September 4th, 2007. URL: http://www.bacterio.cict.fr) Int. J. Syst. Bacteriol. 47:590-592.

Fang, S.W., Yang, C.J., Shih, D.Y., Chou, C.C., and Yu, R.C. 2006. Amplified fragment length polymorphism, serotyping, and quinolone resistance of *Campylobacter jejuni* and *Campylobacter coli* strains from chicken-related samples and humans in Taiwan. J. Food Prot. 69:775-783.

Farber, J.M., and Peterkin, P.I. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55:476-511.

Farber, J.M. 1996. An introduction to the hows and whys of molecular typing. J. Food Prot. 59: 1091-1101.

Fearnley, C., On, S.L.W., Kokotovic, B., Manning, G., Cheasty, T., and Newell, D.G. 2005. Application of fluorescent amplified fragment length polymorphism for comparison of human and animal isolates of *Yersinia enterocolitica*. Appl. Environ. Microbiol. 71:4960-4965.

Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C.V., and Reingold, A.L. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. 312:404-407.

Fonnesbech Vogel, B., Huss, H.H., Ojeniyi, B., Ahrens, P., and Gram, L. 2001. Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. Appl. Environ. Microbiol. 67:2586-2595.

Fonnesbech Vogel, B., Fussing, V., Ojeniyi, B., Gram, L., and Ahrens, P. 2004. High-resolution genotyping of *Listeria monocytogenes* by fluorescent amplified fragment length polymorphism analysis compared to pulsed-field gel electrophoresis, random amplified polymorphic DNA analysis, ribotyping, and PCR-restriction fragment length polymorphism analysis. J. Food Prot. 67:1656-1665.

Fox, G.E., Wisotzkey, J.D., and Jurtshuk, P. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42:166-170.

Franciosa, G., Ferreira, J.L., and Hatheway, C.L. 1994. Detection of type A, B, and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. J. Clin. Microbiol. 32:1911-1917.

Fry, N.K., Bangsborg, J.M., Bernander, S., Etienne, J., Forsblom, B., Gaia, V., Hasenberger, P., Lindsay, D., Papoutsi, A., Pelaz, C., Struelens, M., Uldum, S.A., Visca, P., and Harrison, T.G. 2000. Assessment of intercentre reproducibility and epidemiological concordance of *Legionella pneumophila* serogroup 1 genotyping by amplified fragment length polymorphism analysis. Eur. J. Clin. Microbiol. Infect. Dis. 19:773-780.

Fry, N.K., Bangsborg, J.M., Bergmans, A., Bernander, S., Etienne, J., Franzin, L., Gaia, V., Hasenberger, P., Baladrón Jiménez, B., Jonas, D., Lindsay, D., Mentula, S., Papoutsi, A., Struelens, M., Uldum, S.A., Visca, P., Wannet, W., and Harrison, T.G. 2002. Designation of the European working group on *Legionella* infection (EWGLI) amplified fragment length polymorphism types of *Legionella pneumophila* serogroup 1 and results of intercentre proficiency testing using a standard protocol. Eur. J. Clin. Microbiol. Infect. Dis. 21:722-728.

Fujitani, S., Liu, C.X., Finegold, S.M., Song, Y.L., and Mathisen G.E. 2007. *Clostridium tertium* isolated from gas gangrene wound; misidentified as *Lactobacillus* spp initially due to aerotolerant feature. Anaerobe 13:161-165.

Gaafar, A., Unzaga, M.J., Cisterna, R., Clavo, F.E., Urra, E., Ayarza, R., and Martín, G. 2003. Evaluation of a modified single-enzyme amplified-fragment length polymorphism technique for fingerprinting and differentiating of *Mycobacterium kansasii* type I isolates. J. Clin. Microbiol. 41:3846-3850.

Gandhi, M., and Chikindas, M.L. 2007. *Listeria*: a foodbone pathogen that knows how to survive. Int. J. Food Microbiol. 113:1-15.

García Del Blanco, N., Dobson, M.E., Vela, A.I., De La Puente, V.A., Gutiérrez, C.B., Hadfield, T.L., Kuhnert, P., Frey, J., Domínguez, L., and Rodríguez Ferri, E.F. 2002. Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting and 16S rRNA gene sequencing. J. Clin. Microbiol. 40:2964-2972.

Gebreyes, W.A., and Altier, C. 2002. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolates from swine. J. Clin. Microbiol. 40:2813-2822.

Gebreyes, W.A., Altier, C., and Thakur, S. 2006. Molecular epidemiology and diversity of *Salmonella* serovar Typhimurium in pigs using phenotypic and genotypic approaches. Epidemiol. Infect. 134:187-198.

Geornaras, I., Kunene, N.F., von Holy, A., and Hastings, J.W. 1999. Amplified fragment length polymorphism fingerprinting of *Pseudomonas* strains from a poultry processing plant. Appl. Environ. Microbiol. 65:3828-3833.

Geornaras, I., Hastings, J.W., and von Holy, A. 2001. Genotypic analysis of *Escherichia coli* strains from poultry carcasses and their susceptibilities to antimicrobial agents. Appl. Environ. Microbiol. 67:1940-1944.

Gerner-Smidt, P., Graves, L.M., Hunter, S., and Swaminathan, B. 1998. Computerized analysis of restriction fragment length polymorphism patterns: comparative evaluation of two commercial software packages. J. Clin. Microbiol. 36:1318-1323.

Giammanco, G.M., Mammina, C., Romani, C., Luzzi, I., Dionisi, A.M., and Nastasi, A. 2007. Evaluation of a modified single-enzyme amplified fragment length polymorphism (SE-AFLP) technique for subtyping *Salmonella enterica* serotype Enteritidis. Res. Microbiol. 158:10-17.

Gibson, J.R., Slater, E., Xerry, J., Tompkins, D.S., and Owen, R.J. 1998. Use of an amplified-fragment length polymorphism technique to fingerprint and differentiate isolates of *Helicobacter pylori*. J. Clin. Microbiol. 36:2580-2585.

Gibson, J.R., Ferrus, M.A., Woodward, D., Xerry, J., and Owen, R.J. 1999. Genetic diversity in *Helicobacter pullorum* from human and poultry sources identified by an amplified fragment length polymorphism technique and pulse-field gel electrophoresis. J. Appl. Microbiol. 87:602-610.

Giovannacci, I., Ragimbeau, C., Queguiner, S., Salvat, G., Vendeuvre, J.-L., Carlier, V., and Ermel, G. 1999. *Listeria monocytogenes* in pork slaughtering and cutting plants use of RAPD, PFGE and PCR-REA for tracing and molecular epidemiology. Int. J. Food Microbiol. 53:127-140.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., de Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Ential, K.-E., Fsihi, H., Garcia-Del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Jones, L.-M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueño, E., Maitournam, A., Mata Vicente, J., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Pérez-Diaz, J.-C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vázquez-Boland, J.-A., Voss, H., Wehland, J., and Cossart, P. 2001. Comparative genomics of *Listeria* species. Science 294:849-852.

Godschalk, P.C.R., Bergman, M.P., Gorkink, R.F.J., Simons, G., van den Braak, N., Lastovica, A.J., Endtz, H.P., Verbrugh, H.A., and van Belkum, A. 2006. Identification of DNA sequence variation in *Campylobacter jejuni* strains associated with the Guillain-Barré syndrome by high-throughput AFLP analysis. BMC Microbiol. 6:32.

Goonetilleke, A., and Harris, J.B. 2004. Clostridial neurotoxins. J. Neurol. Neurosurg. Psychiatry 75:35-39.

Goulding, J.N., Hookey, J.V., Stanley, J., Olver, W., Neal, K.R., Ala'aldeen, D.A.A., and Arnold, C. 2000a. Fluorescent amplified-fragment length polymorphism genotyping of *Neisseria meningitidis* identifies clones associated with invasive disease. J. Clin. Microbiol. 38:4580-4585.

Goulding, J.N., Stanley, J., Saunders, N., and Arnold, C. 2000b. Genome-sequence-based fluorescent amplified-fragment length polymorphism analysis of *Mycobacterium tuberculosis*. J. Clin. Microbiol. 38:1121-1126.

Grady, R., Desai, M., O´Neill, G., Cookson, B., and Stanley, J. 1999. Genotyping of epidemic methicillin-resistant *Staphylococcus aureus* phage type 15 isolates by fluorescent amplified-fragment length polymorphism analysis. J. Clin. Microbiol. 37:3198-3203.

Grundmann, H., Hori, S., Enright, M.C., Webster, C., Tami, A., Feil, E.J., and Pitt T. 2002. Determining the genetic structure of the natural population of *Staphylococcus aureus*: a comparison of multilocus sequence typing with pulsed-field gel electrophoresis, randomly amplified polymorphic DNA analysis, and phage typing. J. Clin. Microbiol. 40:4544-4546.

Guan, S., Xu, R., Chen, S., Odumeru, J., and Gyles, C. 2002. Development of a procedure for discriminating among *Escherichia coli* isolates from animal and human sources. Appl. Environ. Microbiol. 68:2690-2698.

Gudbjörnsdóttir, B., Suihko, M.-L., Gustavsson, P., Thorkelsson, G., Salo, S., Sjöberg, A.-M., Niclasen, O., and Bredholt, S. 2004. The incidence of *Listeria monocytogenes* in meat, poultry and seafood plants in the Nordic countries. Food Micribiol. 21:217-225.

Gudmundsdóttir, S., Gudbjörnsdóttir, B., Lauzon, H.L., Einarsson, H., Kristinsson, K.G., and Kristjánsson, M. 2005. Tracing *Listeria monocytogenes* isolates from cold-smoked salmon and its processing environment in Iceland using pulsed-field gel electrophoresis. Int. J. Food Microbiol. 101:41-51.

Gudmundsdóttir, S., Gudbjörnsdóttir, B., Einarsson, H., Kristinsson, K.G., and Kristjánsson, M. 2006. Contamination of cooked peeled shrimp (*Pandalus borealis*) by *Listeria monocytogenes* during processing at two processing plants. J. Food. Prot. 69:1304-1311.

Guerra, M.M., Bernardo, F., and McLauchlin, J. 2002. Amplified fragment length polymorphism (AFLP) analysis of *Listeria monocytogenes*. System. Appl. Microbiol. 25:456-461.

Gzyl, A., Augustynowicz, E., Mosiej, E., Zawadka, M., Gniadek, G., Nowaczek, A., and Slusarczyk, J. 2005. Amplified fragment length polymorphism (AFLP) versus randomly amplified polymorphic DNA (RAPD) as new tools for inter- and intra-species differentiation within *Bordetella*. J. Med. Microbiol. 54:333-346.

Hänninen, M.-L., Perko-Mäkelä, P., Rautelin, H., Duim, B., and Wagenaar, J.A. 2001. Genomic relatedness within five common Finnish *Campylobacter jejuni* pulsed-field gel electrophoresis genotypes studied by amplified fragment length polymorphism analysis, ribotyping, and serotyping. Appl. Environ. Microbiol. 67:1581-1586.

Hatheway, C.L., 1990. Toxigenic Clostridia. Clin. Microbiol. Rev. 3:66-98.

Hatheway, C.L. 1995. Botulism: the present status of the disease. Curr. Top. Microbiol. 195:55-75.

Head, C.B., and Ratnam, S. 1988. Comparison of API ZYM system with API AN-Ident, API 20A, Minitek Anaerobe II, and RapID-ANA systems for identification of *Clostridium difficile*. J. Clin. Microbiol. 26:144-146.

Heikinheimo, A., and Korkeala, H. 2005. Multiplex PCR assay for toxinotyping *Clostridium perfringens* isolates obtained from Finnish broiler chickens. Lett. Appl. Microbiol. 40:407-411.

Heir, E., Lindstedt, B.A., Vardund, T., Wasteson, Y., and Kapperud, G. 2000. Genomic fingerprinting of shigatoxin-producing *Escherichia coli* (STEC) strains: comparison of pulsed-field gel

electrophoresis (PFGE) and fluorescent amplified-fragment-length polymorphism (FAFLP). Epidemiol. Infect. 125:537-548.

Heir, E., Lindstedt, B.-A., Røtterud, O.-J., Vardund, T., Kapperud, G., and Nesbakken, T. 2004. Molecular epidemiology and disinfectant susceptibility of *Listeria monocytogenes* from meat processing plants and human infections. Int. J. Food Microbiol. 96:85-96.

Herrera, S., Cabrera, R., Ramirez, M.M., Usera, M.A., and Echeita, M.A. 2002. Use of AFLP, plasmid typing and phenotyping in a comparative study to assess genetic diversity of *Shigella flexneri* strains. Epidemiol. Infect. 129:445-450.

Hielm, S., Björkroth, J., Hyytiä, E., and Korkeala, H. 1998a. Genomic analysis of *Clostridium botulinum* group II by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. 64:703-708.

Hielm, S., Björkroth, J., Hyytiä, E., and Korkeala, H. 1998b. Prevalence of *Clostridium botulinum* in Finnish trout farms: pulsed-field gel electrophoresis typing reveals extensive genetic diversity among type E isolates. Appl. Environ. Microbiol. 64:4161-4167.

Hill, K.K., Ticknor, L.O., Okinaka, R.T., Asay, M., Blair, H., Bliss, K.A., Laker, M., Pardington, P.E., Richardson, A.P., Tonks, M., Beecher, D.J., Kemp, J.D., Kolstø, A.-B., Lee Wong, A.C., Keim, P., and Jackson, P.J. 2004. Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. 70:1068-1080.

Hill, K.K., Smith, T.J., Helma, C.H., Ticknor, L.O., Foley, B.T., Svensson, R.T., Brown, J.L., Johnson, E.A., Smith, L.A., Okinaka, R.T., Jackson, P.J., and Marks, J.D. 2007. Genetic diversity among botulinum neurotoxin-producing clostridial strains. J. Bacteriol. 189:818-832.

Hoffman, A.D., Gall, K.L., Norton, D.M., and Wiedmann, M. 2003. *Listeria monocytogenes* contamination patterns for the smoked fish processing environment and for raw fish. J. Food Prot. 66:52-60.

Holah, J.T., Bird, J., and Hall, K.E. 2004. The microbial ecology of high-risk, chilled food factories; evidence for persistent *Listeria* spp., and *Escherichia coli* strains. J. Appl. Microbiol. 97:68-77.

Holdeman, L.V., Cato, E.P., and Moore, W.E.C. 1977. Anaerobe laboratory manual, 4th edition. Anaerobe Laboratory, The Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Homan, W.L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., van Embden, J.D.A., and Willems, R.J.L. 2002. Multilocus sequence typing scheme for *Enterococcus faecium*. J. Clin. Microbiol. 40:1963-1971.

Hong, Y., García, M., Levisohn, S., Lysnyansky, I., Leiting, V., Savelkoul, P.H.M., and Kleven, S.H. 2005. Evaluation of amplified fragment length polymorphism for differentiation of avian mycoplasma species. J. Clin. Microbiol. 43:909-912.

Hookey, J.V., Edwards, V., Patel, S., Richardson, J.F., and Cookson, B.D. 1999. Use of fluorescent amplified fragment length polymorphism (fAFLP) to characterise methicillin-resistant *Staphylococcus aureus*. J. Microbiol. Meth. 37:7-15.

Hu, H., Lan, R., and Reeves, P.R. 2002. Fluorescent amplified fragment length polymorphism analysis of *Salmonella enterica* serovar Typhimurium reveals phage-type-specific markers and potential for microarray typing. J. Clin. Microbiol. 40:3406-3415.

Hu, Y., Gall, K., Ho, A., Ivanek, R., Gröhn, Y.T., and Wiedmann, M. 2006. Daily variability of *Listeria* contamination patterns in a cold-smoked salmon processing operation. J. Food Prot. 69:2123-2133.

Huber, B.S., Allred, D.V., Carmen, J.C., Frame, D.D., Whiting, D.G., Cryan, J.R., Olson, T.R., Jackson, P.J., Hill, K., Laker, M.T., and Robison, R.A. 2002. Random amplified polymorphic DNA

and amplified fragment length polymorphism analyses of *Pasteurella multocida* isolates from fatal fowl cholera infections. J. Clin. Microbiol. 40:2163-2168.

Hunter, P.R., and Gaston, M.A. 1998. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26:2465-2466.

Hutson, R.A., Thompson, D.E., Lawson, P.A., Schocken-Itturino, R.P., Böttger, E.C., and Collins, M.D. 1993. Genetic interrelationships of proteolytic *Clostridium botulinum* types A, B, and F and other members of the *Clostridium botulinum* complex as revealed by small-subunit rRNA gene sequences. Antonie Leeuwenhoek 64:273-283.

Huys, G., Coopman, R., Janssen, P., and Kersters, K. 1996a. High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. Int. J. Syst. Bacteriol. 46:572-580.

Huys, G., Kersters, I., Coopman, R., Janssen, P., and Kersters, K. 1996b. Genotypic diversity among *Aeromonas* isolates recovered from drinking water production plants as revealed by AFLP analysis. System. Appl. Microbiol. 19:428-435.

Huys, G., Rigouts, L., Chemlal, K., Portaels, F., and Swings, J. 2000. Evaluation of amplified fragment length polymorphism analysis for inter- and intraspecific differentiation of *Mycobacterium bovis*, *M. tuberculosis*, and *M. ulcerans*. J. Clin. Microbiol. 38:3675-3680.

Hyytiä, E., Björkroth, J., Hielm, S., and Korkeala, H. 1999a. Characterisation of *Clostridium botulinum* groups I and II by randomly amplified polymorphic DNA analysis and repetitive element sequence-based PCR. Int. J. Food Microbiol. 48:179-189.

Hyytiä, E., Hielm, S., Björkroth, J., and Korkeala, H. 1999b. Biodiversity of *Clostridium botulinum* type E strains isolated from fish and fishery products. Appl. Environ. Microbiol. 65:2057-2064.

Imataki, O., Makimoto, A., Kato, S., Bannai, T., Numa, N., Nukui, Y., Morisawa, Y., Ishida, T., Kami, M., Fukuda, T., Mori, S., Tanosaki, R., and Takaue, Y. 2006. Coincidental outbreak of methicillin-resistant *Staphylococcus aureus* in a hematopoietic stem cell transplantation unit. Am. J. Hematol. 81:664-669.

Ip, M., Lyon, D.J., Chio, F., Enright, M.C., and Cheng, A.F. 2003. Characterization of isolates of methicillin-resistant *Staphylococcus aureus* from Hong Kong by phage typing, pulsed-field gel electrophoresis, and fluorescent amplified-fragment length polymorphism analysis. J. Clin. Microbiol. 41:4980-4985.

Iyoda, S., Wada, A., Weller, J., Flood, S.J.A., Schreiber, E., Tucker, B., and Watanabe, H. 1999. Evaluation of AFLP, a high-resolution DNA fingerprinting method, as a tool for molecular subtyping of enterohemorrhagic *Escherichia coli* O157:H7 isolates. Microbiol. Immunol. 43:803-806.

Izumiya, H., Terajima, J., Wada, A., Inagaki, Y., Itoh, K.-I., Tamura, K., and Watanabe, H. 1997. Molecular typing of enterohemorrhagic *Escherichia coli* O157:H7 isolates in Japan by using pulsed-field gel electrophoresis. J. Clin. Microbiol. 35:1675-1680.

Jackson, P.J., Hill, K.K., Laker, M.T., Ticknor, L.O., and Keim. P. 1999. Genetic comparison of *Bacillus anthracis* and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis. J. Appl. Microbiol. 87:263-269.

Jaimes, C.P., Aristizábal, F.A.G., Bernal, M.M., Suárez, Z.R., and Montoya, D. 2006. AFLP fingerprinting of Colombian *Clostridium* spp strains, multivariate data analysis and its taxonomical implications. J. Microbiol. Meth. 67:64-69.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M., and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142:1881-1893.

Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K., and Dijkshoorn, L. 1997. Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting. Int. J. Syst. Bacteriol. 47:1179-1187.

Johannessen, G.S., Johnsen, G., Økland, M., Cudjoe, K.S., and Hofshagen, M. 2007. Enumeration of thermotolerant *Campylobacter* spp. from poultry carcasses at the end of the slaughter-line. Lett. Appl. Microbiol. 44:92-97.

Johansson, T., Rantala, L., Palmu, L., and Honkanen-Buzalski, T. 1999. Occurrence and typing of *Listeria monocytogenes* strains in retail vacuum-packed fish products and in a production plant. Int. J. Food Microbiol. 47:111-119.

Johnsen, G., Kruse, H., and Hofshagen, M. 2006a. Genetic diversity and description of transmission routes for *Campylobacter* on broiler farms by amplified-fragment length polymorphism. J. Appl. Microbiol. 101:1130-1139.

Johnsen, G., Kruse, H., and Hofshagen, M. 2006b. Genotyping of *Campylobacter jejuni* from broiler carcasses and slaughterhouse environment by amplified fragment length polymorphism. Poultry Sci. 85:2278-2284.

Johnsen, G., Zimmerman, K., Lindstedt, B.A., Vardund, T., Herikstad, H., and Kapperud, G. 2006c. Intestinal carriage of *Campylobacter jejuni* and *Campylobacter coli* among cattle from South-western Norway and comparative genotyping of bovine and human isolates by amplified-fragment length polymorphism. Acta Vet. Scand. 48:4.

Johnson, J.L., and Francis, B.S. 1975. Taxonomy of the clostridia: ribosomal ribonucleic acid homologies among the species. J. Gen. Microbiol. 88:229-244.

Jonas, D., Meyer, H.-G.W., Matthes, P., Hartung, D., Jahn, B., Daschner, F.D., and Jansen, B. 2000. Comparative evaluation of three different genotyping methods for investigation of nosocomial outbreaks of Legionnaires' disease in hospitals. J. Clin. Microbiol. 38:2284-2291.

Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, F., van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A., and Karp, A. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol. Breeding 3:381-390.

Jureen, R., Harthug, S., Sørnes, S., Digranes, A., Willems, R.J.L., and Langeland, N. 2004. Comparative analysis of amplified fragment length polymorphism and pulsed field gel electrophoresis in a hospital outbreak and subsequent endemicity of ampicillin-resistant *Enterococcus faecium*. FEMS Immunol. Med. Mic. 40:33-39.

Kassama, Y., Rooney, P.J., and Goodacre, R. 2002. Fluorescent amplified fragment length polymorphism probabilistic database for identification of bacterial isolates from urinary tract infections. J. Clin. Microbiol. 40:2795-2800.

Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S.E., Richmond, K., Adair, D.M., Hugh-Jones, M., Kuske, C.R., and Jackson, P. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. 179: 818-824.

Keller, J., Wieland, B., Wittwer, M., Stephan, R., and Perreten, V. 2007. Distribution and genetic variability among *Campylobacter* spp. isolates from different animal species and humands in Switzerland. Zoonoses Public health. 54:2-7.

Kennett, C.A., and Stark, B. 2006. Automated ribotyping for the identification and characterization of foodborne clostridia. J. Food Prot. 69:2970-2975.

Kiehnbaum, L.A., Amonsin, A., Wells, S.J., and Kapur, V. 2005. Amplified fragment length polymorphism to detect clonal diversity and distribution of *Mycobacterium avium* subspecies *paratuberculosis* in selected Minnesota dairy cattle. J. Vet. Diagn. Invest. 17:311-315.

Killgore, G., Thompson, A., Johnson, S., Brazier, J., Kuijper, E., Pepin, J., Frost, E.H., Savelkoul, P., Nicholson, B., van den Berg, R.J., Kato, H., Sambol, S.P., Zukowski, W., Woods, C., Limbago, B., Gerding, D.N., and McDonald, L.C. 2008. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. J. Clin. Microbiol. 46:431-437.

Kitch, T.T., and Appelbaum, P.C. 1989. Accuracy and reproducibility of the 4-hour ATB 32A method for anaerobe identification. J. Clin. Microbiol. 27:2509-2513.

Kivioja, T., Arvas, M., Saloheimo M., Penttilä, M., and Ukkonen, E. 2005. Optimization of cDNA-AFLP experiments using genomic sequence data. Bioinformatics 21:2573-2579.

Klaassen, C.H.W., van Haren, H.A., and Horrevorts, A.M. 2002. Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. J. Clin. Microbiol. 40:101-104.

Klijn, N., Nieuwenhof, F.J., Hoolwerf, J.D., van der Waals, C.B., and Weerkamp, A.H. 1995. Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplificiation. Appl. Environ. Microbiol. 61:2919-2924.

Knoop, F.C., Owens, M., and Crocker, I.C. 1993. *Clostridium difficile*: clinical disease and diagnosis. Clin. Microbiol. Rev. 6:251-265.

Koeleman, J.G.M., Stoof, J., Biesmans, D.J., Savelkoul, P.H.M., and Vandenbroucke-Grauls, C.M.J.E. 1998. Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii*. J. Clin. Microbiol. 36:2522-2529.

Kokotovic, B., Friis, N.F., Jensen, J.S., and Ahrens, P. 1999. Amplified-fragment length polymorphism fingerprinting of *Mycoplasma* species. J. Clin. Microbiol. 37:3300-3307.

Kokotovic, B., and On, S.L.W. 1999. High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphism. FEMS Microbiol. Lett. 173:77-84.

Kristjánsson, M., Samore, M.H., Gerding, D.N., DeGirolami, P.C., Bettin, K.M., Karchmer, A.W., and Arbeit, R.D. 1994. Comparison of restriction endonuclease analysis, ribotyping, and pulsed-field gel electrophoresis for molecular differentiation of *Clostridium difficile* strains. J. Clin. Microbiol. 32:1963-1969.

Kusiluka, L.J.M., Ojeniyi, B., Friis, N.F., Kokotovic, B., and Ahrens, P. 2001. Molecular analysis of field strains of *Mycoplasma capricolum* subspecies *capripneumoniae* and *Mycoplasma mycoides* subspecies *mycoides*, small colony type isolated from goats in Tanzania. Vet. Microbiol. 82: 27-37.

La Rosa, G., De Carolis, E., Sali, M., Papacchini, M., Riccardi, C., Mansi, A., Paba, E., Alquati, C., Bestetti, G., and Muscillo, M. 2006. Genetic diversity of bacterial strains isolated from soils, contaminated with polycyclic aromatic hydrocarbons, by 16S rRNA gene sequencing and amplified fragment length polymorphism fingerprinting. Microbiol. Res. 161:150-157.

Lahti, P., Heikinheimo, A., Johansson, T., and Korkeala, H. 2008. *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS*1151*-*cpe* or IS*1470*-like-*cpe*) as a common cause of food poisoning. J. Clin. Microbiol. 46:371-373.

Lan, R., and Reeves, P.R. 2002. Pandemic spread of cholera: genetic diversity and relationships within the seventh pandemic clone of *Vibrio cholerae* determined by amplified fragment length polymorphism. J. Clin. Microbiol. 40:172-181.

Lappi, V.R., Thimothe, J., Walker, J., Bell, J., Gall, K., Moody, M.W., and Wiedmann, M. 2004. Impact of intervention strategies on *Listeria* contamination patterns in crawfish processing plants: a longitudinal study. J. Food Prot. 67:1163-1169.

Lau, S.K.P., Ng, K.H.L., Woo, P.C.Y., Yip, K.T., Fung, A.M.Y., Woo, G.K.S., Chan, K.M., Que, T.L., and Yuen, K.Y. 2006. Usefulness of the MicroSeq 500 16S rDNA bacterial identification system for identification of anaerobic Gram positive bacilli isolated from blood cultures. J. Clin. Pathol. 59:219-222.

Lavigne, J.-P., Bouziges, N., Sotto, A., Leroux, J.-L., and Michaux-Charachon, S. 2003. Spondylodiscitis due to *Clostridium ramosum* infection in an immunocompetent elderly patient. J. Clin. Microbiol. 41:2223-2226.

Lawrence, L.M., and Gilmour, A. 1995. Characterization of *Listeria monocytogenes* isolated from poultry products and from the poultry-processing environment by random amplification of polymorphic DNA and multilocus enzyme electrophoresis. Appl. Environ. Microbiol. 61:2139-2144.

Lawson, A.J., Stanley, J., Threlfall, E.J., and Desai, M. 2004. Fluorescent amplified fragment length polymorphism subtyping of multiresistant *Salmonella enterica* serovar Typhimurium DT104. J. Clin. Microbiol. 42:4843-4845.

Lawson, P.A., Llop-Perez, P., Hutson, R.A., Hippe, H., and Collins, M.D. 1993. Towards a phylogeny of the clostridia based on 16S rRNA sequences. FEMS Microbiol. Lett. 113:87-92.

Lee, W.H., and Riemann, H. 1970. The genetic relatedness of proteolytic *Clostridium botulinum* strains. J. Gen. Microbiol. 64:85-90

Lewis, C.J., and Naylor, R.D. 1998. Sudden death in sheep associated with *Clostridium sordellii*. Vet. Rec. 142:417-421.

Lilly, T., Harmon, S.M., Kautter, D.A., Solomon, H.M., and Lynt, R.K. 1971. An improved medium for detection of *Clostridium botulinum* type E. J. Milk Food Technol. 34: 492-497.

Lin, J.J., Ma, J., and Kuo, J. 1999. Chemiluminescent detection of AFLP markers. BioTechniques 26:344-348.

Lindstedt, B.A., Heir, E., Vardund, T., and Kapperud, G. 2000a. Fluorescent amplified-fragment length polymorphism genotyping of *Salmonella enterica* subsp. *enterica* serovars and comparison with pulsed-field gel electrophoresis typing. J. Clin. Microbiol. 38:1623-1627.

Lindstedt, B.A., Heir, E., Vardund, T., and Kapperud, G. 2000b. A variation of the amplified fragment length polymorphism (AFLP) technique using three restriction endonucleases, and assessment of the enzyme combination *Bgl*II-*Mfe*I for AFLP analysis of *Salmonella enterica* subsp. *enterica* isolates. FEMS Microbiol. Lett. 189:19-24.

Lindstedt, B.A., Heir, E., Vardund, T., Melby, K.K., and Kapperud, G. 2000c. Comparative fingerprinting analysis of *Campylobacter jejuni* subsp. j*ejuni* by amplified-fragment length polymorphism genotyping. J. Clin. Microbiol. 38:3379-3387.

Lindstedt, B.A., Heir, E., Gjernes, E., and Kapperud, G. 2003. DNA fingerprinting of *Salmonella enterica* subsp. *enterica* serovar Typhimurium with emphasis on phage type DT104 based on variable number of tandem repeat loci. J. Clin. Microbiol. 41:1469-1479.

Lindström, M.K., Jankola, H.M., Hielm, S., Hyytiä, E.K., and Korkeala, H.J. 1999. Identification of *Clostridium botulinum* with API 20 A, Rapid ID 32 A and RapID ANA II. FEMS Immunol. Med. Mic. 24:267-274.

Lindström, M., Keto, R., Markkula, A., Nevas, M., Hielm, S., and Korkeala, H. 2001. Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. Appl. Environ. Microbiol. 67:5694-5699.

Lindström, M., Kiviniemi, K., and Korkeala H. 2006. Hazard and control of group II (non-proteolytic) *Clostridium botulinum* in modern food processing. Int. J. Food Microbiol. 108:92-104.

Lindström, M., and Korkeala, H. 2006. Laboratory diagnostics of botulism. Clin. Microbiol. Rev. 19:298-314.

Looney, W.J., Gallusser, A.J.C., and Modder, H.K. 1990. Evaluation of the ATB 32 A system for identification of anaerobic bacteria isolated from clinical specimens. J. Clin. Microbiol. 28:1519-1524.

López, V., Ortiz, S., Corujo, A., López, P., Navas, J., Moreno, R., and Martínez-Suárez, J.V. 2007. Traceback identification of an ingredient (pork dewlap) as the possible source of *Listeria monocytogenes* serotype 4b contamination in raw chicken products. J. Food Prot. 70:1513-1517.

Low, J.C., and Donachie, W. 1997. A review of *Listeria monocytogenes* and listeriosis. Vet. J. 153:9-29.

Lukinmaa, S., Takkunen, E., and Siitonen, A. 2002. Molecular epidemiology of *Clostridium perfringens* related to food-borne outbreaks of disease in Finland from 1984-1999. Appl. Environ. Microbiol. 68:3744-3749.

Lundén, J.M., Miettinen, M.K., Autio, T.J., and Korkeala, H.J. 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. J. Food Prot. 63:1204-1207.

Lundén, J.M., Autio, T.J., and Korkeala, H.J. 2002. Transfer of persistent *Listeria monocytogenes* contamination between food-processing plants associated with a dicing machine. J. Food Prot. 65:1129-1133.

Lundén, J., Autio, T., Markkula, A., Hellström, S., and Korkeala, H. 2003a. Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. Int. J. Food Microbiol. 82:265-272.

Lundén, J.M., Autio, T.J., Sjöberg, A.-M., and Korkeala, H.J. 2003b. Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. J. Food Prot. 66:2062-2069.

Lynch, M., Painter, J., Woodruff, R., and Braden, C. 2006. Surveillance for foodborne-disease outbreaks – United states, 1998-2002. MMWR Surveill. Summ 55:1-34.

Lynt, R.K., Kautter, D.A., and Solomon, H.M. 1982. Differences and similarities among proteolytic and nonproteolytic strains of *Clostridium botulinum* types A, B, E and F: a review. J. Food. Prot. 45:466-474.

Lyytikäinen, O., Autio, T., Maijala, R., Ruutu, P., Honkanen-Buzalski, T., Miettinen, M., Hatakka, M., Mikkola, J., Anttila, V.-J., Johansson, T., Rantala, L., Aalto, T., Korkeala, H., and Siitonen, A. 2000. An outbreak of *Listeria monocytogenes* serotype 3a from butter in Finland. J. Infect. Dis. 181:1838-1841.

Macdonald, T.E., Helma, C.H., Ticknor, L.O., Jackson, P.J., Okinaka, R.T., Smith, L.A., Smith, T.J., and Hill, K.K. 2008. Differentiation of *Clostridium botulinum* serotype A strains using multiple-locus variable-number tandem-repeat analysis. Appl. Environ. Microbiol. 74:875-882.

Markkula, A., Autio, T., Lundén, J., and Korkeala, H. 2005. Raw and processed fish show identical *Listeria monocytogenes* genotypes with pulsed-field gel electrophoresis. J. Food Prot. 68:1228-1231.

Marler, L.M., Siders, J.A., Wolters, L.C., Pettigrew, Y., Skitt, B.L., and Allen, S.D. 1991. Evaluation of the new RapID-ANA II system for the identification of clinical anaerobic isolates. J. Clin. Microbiol. 29:874-878.

Marshall, S., Clark, C.G., Wang, G., Mulvey, M., Kelly, M.T., and Johnson, W.M. 1999. Comparison of molecular methods for typing *Vibrio parahaemolyticus*. J. Clin. Microbiol. 37:2473-2478.

Martinez, I., Rørvik, L.-M., Brox, V., Lassen, J., Seppola, M., Gram, L., and Fonnesbech-Vogel, B. 2003. Genetic variability among isolates of *Listeria monocytogenes* from food products, clinical samples and processing environments, estimated by RAPD typing. Int. J. Food Microbiol. 84:285-297.

Maslow, J.N., Mulligan, M.E., and Arbeit, R.D. 1993. Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. Clin. Infect. Dis. 17:153-164.

McLauchlin, J., and Low, J.C. 1994. Primary cutaneous listeriosis in adults: an occupational disease of veterinarians and farmers. Vet. Rec. 135:615-617.

McLauchlin, J., Ripabelli, G., Brett, M.M., and Threlfall, E.J. 2000. Amplified fragment length polymorphism (AFLP) analysis of *Clostridium perfringens* for epidemiological typing. Int. J. Food Microbiol. 56:21-28.

McLauchlin, J., Salmon, J.E., Ahmed, S., Braxier, J.S., Brett, M.M., George, R.C., and Hood, J. 2002. Amplified fragment length polymorphism (AFLP) analysis of *Clostridium novyi*, *C. perfringens* and *Bacillus cereus* isolated from injecting drug users during 2000. J. Med. Microbiol. 51:990-1000.

Meays, C.L., Broersma, K., Nordin, R., and Mazumder, A. 2004. Source tracking fecal bacteria in water: a critical review of current methods. J. Environ. Manage. 73:71-79.

Melles, D.C., Gorkink, R.F.J., Boelens, H.A.M., Snijders, S.V., Peeters, J.K., Moorhouse, M.J., van der Spek, P.J., van Leeuwen, W.B., Simons, G., Verbrugh, H.A., and van Belkum, A. 2004. Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. J.Clin. Invest. 114:1732-1740.

Melles, D.C., van Leeuwen, W.B., Snijders, S.V., Horst-Kreft, D., Peeters, J.K., Verrugh, H.A., and van Belkum A. 2007. Comparison of multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for genetic typing of *Staphylococcus aureus*. J. Microbiol. Meth. 69:371-375.

Miettinen, M.K., Björkroth, K.J., and Korkeala, H. 1999a. Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. Int. J. Food Microbiol. 46:187-192.

Miettinen, M.K., Siitonen, A., Heiskanen, P., Haajanen, H., Björkroth, K.J., and Korkeala, H.J. 1999b. Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. J. Clin. Microbiol. 37:2358-2360.

Mikasová, E., Drahovská, H., Szemes, T., Kuchta, T., Karpíšková, R., Sásik, M., and Turňa, J. 2005a. Characterization of *Salmonella enterica* serovar Typhimurium strains of veterinary origin by molecular typing methods. Vet. Microbiol. 109:113-120.

Mikasová, E., Oravcová, K., Kaclíková, E., Kuchta, T., and Drahovská, H. 2005b. Typing of food-borne *Listeria monocytogenes* by polymerase chain reaction – restriction enzyme analysis and amplified fragment length polymorphism. New Microbiol. 28:265-270.

Mikkonen, T., Koort, J.M.K., Björkroth, K.J., and Sukura, A. 2005. Testing of amplified fragment length polymorphism (AFLP) technique as a tool for molecular epidemiology of *Trichinella nativa*. Vet. Parasitol. 132:19-22.

Moran, N.A. 2002. Microbial minimalism: genome reduction in bacterial pathogens. Cell 108:583-586.

Mortimer, P., and Arnold, C. 2001. FAFLP: last word in microbial genotyping? J. Med. Microbiol. 50:393-395.

Motiwala, A.S., Strother, M., Amonsin, A., Byrum, B., Naser, S.A., Stabel, J.R., Shulaw, W.P., Bannantine, J.P., Kapur, V., and Sreevatsan, S. 2003. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: evidence for limited strain diversity, strain sharing, and identification of unique targets for diagnosis. J. Clin. Microbiol. 41:2015-2026.

Mougel, C., Thioulouse, J., Perrière, G., and Nesme, X. 2002. A mathematical method for determining genome divergence and species delineation using AFLP. Int J. Syst. Evol. Micr. 52:573-586.

Mueller, U.G., and Wolfenbarger, L.L. 1999. AFLP genotyping and fingerprinting. Trends Ecol. Evol. 14:389-394.

Nair, S., Schreiber, E., Thong, K.L., Pang, T., and Altwegg, M. 2000. Genotypic characterization of *Salmonella typhi* by amplified fragment length polymorphism fingerprinting provides increased discrimination as compared to pulsed-field gel electrophoresis and ribotyping. J. Microbiol. Meth. 41:35-43.

Nakamura, H., Tokuda, Y., Sono, A., Koyama, T., Ogasawara, J., Hase, A., Haruki, K., and Nishikawa, Y. 2006. Molecular typing to trace *Listeria monocytogenes* isolated from cold-smoked fish to a contamination source in a processing plant. J. Food Prot. 69:835-841.

Neeleman, C., Klaassen, C.H.W., de Valk, H.A., de Ruiter, M.T., and Mouton, J.W. 2004. Amplified fragment length polymorphism fingerprinting is an effective technique to distinguish *Streptococcus pneumoniae* from other streptococci and an efficient alternative to pulsed-field gel elctrophoresis for molecular typing of pneumococci. J. Clin. Microbiol. 42:369-371.

Nesbakken, T., Kapperud, G., and Caugant, D.A. 1996. Pathways of *Listeria monocytogenes* contamination in the meat processing industry. Int. J. Food Microbiol. 31:161-171.

Norton, D.M., McCamey, M.A., Gall, K.L., Scarlett, J.M., Boor, K.J., and Wiedmann, M. 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. Appl. Environ. Microbiol. 67:198-205.

Norwood, D.E., and Gilmour, A. 1999. Adherence of *Listeria monocytogenes* strains to stainless steel coupons. J. Appl. Microbiol. 86:576-582.

Nørrung, B., Andersen, J.K., and Schlundt, J. 1999. Incidence and control of *Listeria monocytogenes* in foods in Denmark. Int. J. Food Microbiol. 53:195-203.

Olive, D.M., and Bean, P. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. 37:1661-1669.

On, S.L.W., and Harrington, C.S. 2000. Identification of taxonomic and epidemiological relationships among *Campylobacter* species by numerical analysis of AFLP profiles. FEMS Microbiol. Lett. 193:161-169.

On, S.L.W., Harrington, C.S., and Atabay, H.I. 2003. Differentiation of *Arcobacter* species by numerical analysis of AFLP profiles and description of a novel *Arcobacter* from pig abortions and turkey faeces. J. Appl. Microbiol. 95:1096-1105.

On, S.L.W., Atabay, H.I., Amisu, K.O., Coker, A.O., and Harrington, C.S. 2004. Genotyping and genetic diversity of *Arcobacter butzleri* by amplified fragment length polymorphism (AFLP) analysis. Lett. Appl. Microbiol. 39:347-352.

O'Shea, B., Khare, S., Bliss, K., Klein, P., Ficht, T.A., Adams, L.G., and Rice-Ficht, A.C. 2004. Amplified fragment length polymorphism reveals genomic variability among *Mycobaterium avium* subsp. *paratuberculosis* isolates. J. Clin. Microbiol. 42:3600-3606.

Pattanayak, D., Srinivasan, K., Mandaokar, A.D., Shukla, A., Bhalla, R., and Kumar, P.A. 2000. AFLP fingerprinting and genotypic characterization of some serovars of *Bacillus thuringiensis*. World J. Microb. Biot. 16:667-672.

Pearson, K. 1926. On the coefficient of racial likeness. Biometrika 18:105-117.

Petit, L., Gibert, M., and Popoff, M.R. 1999. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol. 7:104-110.

Picardeau, M., Prod'hom, G., Raskine, L., LePennec, M.P., and Vincent, V. 1997. Genotypic characterization of five subspecies of *Mycobacterium kansasii*. J. Clin. Microbiol. 35:25-32.

Pitcher, D.G., Saunders, N.A., and Owen, R.J. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8:151-156.

Portier, P., Fischer-Le Saux, M., Mougel, C., Lerondelle, C., Chapulliot, D., Thioulouse, J., and Nesme, X. 2006. Identification of genomic species in *Agrobacterium* biovar 1 by AFLP genomic markers. Appl. Environ. Microbiol. 72:7123-7131.

Power, E.G.M: 1996. RAPD typing in microbiology; a technical review. J. Hosp. Infect. 34:247-265.

Rademaker, J.L.W., Hoste, B., Louws, F.J., kersters, K., Swings J., Vauterin, L. Vauterin, P., and de Bruijn, F.J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. Int. J. Syst. Evol. Micr. 50:665-677.

Radnedge, L., Agron, P.G., Hill, K.K., Jackson, P.J., Ticknor, L.O., Keim, P., and Andersen, G.L. 2003. Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. Appl. Environ. Microbiol. 69:2755-2764.

Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P., and Vijila, H.M. 2007. *Listeria* – review of epidemiology and pathogenesis. J. Microbiol. Immunol. Infect. 40:4-13.

Rasmussen, O.F., Skouboe, P., Dons, L., Rossen, L., and Olsen, J.E. 1995. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. Microbiology 141:2053-2061.

Reche, M.P., Echeita, M.A., García de los Rios, J.E., Usera, M.A., Jiménez, P.A., Rojas, A.M., Colás, J., and Rodriguez, I. 2003. Comparison of phenotypic and genotypic markers for characterization of an outbreak of *Salmonella* serotype Havana in captive raptors. J. Appl. Microbiol. 94:65-72.

Rehm, T., Baums, C.G., Strommenger, B., Beyerbach, M., Valentin-Weigand, P., and Goethe, R. 2007. Amplified fragment length polymorphism of *Streptococcus suis* strains correlates with their profile of virulence-associated genes and clinical background. J. Med. Microbiol. 56:102-109.

Reijans, M., Lascaris, R., Groeneger, A.O., Wittenberg, A., Wesselink, E., van Oeveren, J., de Wit, E., Boorsma, A., Voetdijk, B., van der Spek, H., Grivell, L.A., and Simons, G. 2003. Quantitative comparison of cDNA-AFLP, microarrays and GeneChip expression data in *Saccharomyces cerevisiae*. Genomics 82:606-618.

Riedo, F.X., Pinner, R.W., Tosca, M.L., Cartter, M.L., Graves, L.M., Reeves, M.W., Weaver, R.E., Plikaytis, B.D., and Broome, C.V. 1994. A point-source foodborne listeriosis outbreak: documented incubation period and possible mild illness. J. Infect. Dis. 170:693-696.

Ripabelli, G., McLauchlin, J., Mithani, V., and Threlfall, E.J. 2000a. Epidemiological typing of *Bacillus cereus* by amplified fragment length polymorphism. Lett. Appl. Microbiol. 30:358-363.

Ripabelli, G., McLauchlin, J., and Threlfall, E.J. 2000b. Amplified fragment length polymorphism (AFLP) analysis of *Listeria monocytogenes*. System. Appl. Microbiol. 23: 132-136.

Roblot, P., Roblot, F., Fauchére, J.L., Devilleger, A., Maréchaud, R., Breux, J.P., Grollier, G., and Becq-Giraudon, B. 1994. Retrospective study of 108 cases of botulism in Poitiers, France. J. Med. Microbiol. 40:379-384.

Rombauts, S., Van de Peer, Y., and Rouzé, P. 2003. AFLPinSilico, simulating AFLP fingerprints. Bioinformatics 19:776-777.

Rood, J.I., and Cole, S.T. 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol. Rev. 55:621-648.

Rudolf, M., and Scherer, S. 2001. High incidence of *Listeria monocytogenes* in European red smear cheese. Int. J. Food Microbiol. 63:91-98.

Ruiz, M., Rodríguez, J.C., Rodríguez-Valera, F., and Royo, G. 2003. Amplified-fragment length polymorphism as a complement to IS*6110*-based restriction fragment length polymorphism analysis for molecular typing of *Mycobacterium tuberculosis.* J. Clin. Microbiol. 41:4820-4822.

Ryu, C., Lee, K., Hawng, H.J., Yoo, C.K., Seong, W.K., and Oh, H.B. 2005. Molecular characterization of Korean *Bacillus anthracis* isolates by amplified fragment length polymorphism analysis and multilocus variable-number tandem repeat analysis. Appl. Environ. Microbiol. 71:4664-4671.

Rørvik, L.M., Caugant, D.A., and Yndestad, M. 1995. Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. Int. J. Food Microbiol. 25:19-27.

Rørvik, L.M., Skjerve, E., Knudsen, B.R., and Yndestad, M. 1997. Risk factors for contamination of smoked salmon with *Listeria monocytogenes* during processing. Int. J. Food Microbiol. 37:215-219.

Rørvik, L.M. 2000. *Listeria monocytogenes* in the smoked salmon industry. Int. J. Food Microbiol. 62:183-190.

Rørvik, L.M., Aase, B., Alvestad, T., and Caugant, D.A. 2003. Molecular epidemiological survey of *Listeria monocytogenes* in broilers and poultry products. J. Appl. Microbiol. 94:633-640.

Sauders, B.D. Durak, M.Z., Fortes, E., Windham, K., Schukken, Y., Lembo Jr., A.J., Akey, B., Nightingale, K.K., and Wiedmann, M. 2006. Molecular characterization of *Listeria monocytogenes* from natural and urban environments. J. Food Prot. 69:93-105.

Savelkoul, P.H.M., Aarts, H.J.M., De Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J.L.W., Schouls, L., and Lenstra, J.A. 1999. Amplified-fragment length polymorphism analysis: the state of an art. J. Clin. Microbiol. 37:3083-3091.

Sawabe, T., Thompson, F.L., Heyrman, J., Cnockaert, M., Hayashi, K., Tanaka, R., Yoshimizu, M., Hoste, B., Swings, J., and Ezura, Y. 2002. Fluorescent amplified fragment length polymorphism and repetitive extragenic palindrome-PCR fingerprinting reveal host-specific genetic diversity of *Vibrio halioticoli*-like strains isolated from the gut of Japanese abalone. Appl. Environ. Microbiol. 68:4140-4144.

Sawires, Y.S., and Songer, J.G. 2006. *Clostridium perfringens*: insight into virulence evolution and population structure. Anaerobe 12:23-43.

Schalch, B., Bader, L., Schau, H.P., Bergmann, R., Rometsch, A., Maydl, G., and Keßler, S. 2003. Molecular typing of *Clostridium perfringens* from a food-borne disease outbreak in a nursing home: ribotyping versus pulsed-field gel electrophoresis. J. Clin. Microbiol. 41:892-895.

Schlech, W.F., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., and Broome, C.V. 1983. Epidemic listeriosis: evidence for transmission by food. N. Engl. J. Med. 308:203-206.

Schouls, L.M., Reulen, S., Duim, B., Wagenaar, J.A., Willems, R.J.L., Dingle, K.E., Colles, F.M., and Van Embden, J.D.A. 2003. Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: strain diversity, host range, and recombination. J. Clin. Microbiol. 41:15-26.

Scott, F., Threlfall, J., Stanley, J., and Arnold, C. 2001. Fluorescent amplified fragment length polymorphism genotyping of *Salmonella* Enteritidis: a method suitable for rapid outbreak recognition. Clin. Microbiol. Infect. 7:479-485.

Seeliger, H.P.R., and Jones, D. 1986. Genus *Listeria*. In Bergeys Manual of Systematic Bacteriology, Vol. 2. Sneath, P.H.A., Mair, N.S., and Sharpe, M.E. *et al*. (eds). Williams & Wilkins, Baltimore, pp. 1235-1245.

Shaaly, A., Tellevik, M.G., Langeland, N., Høiby, E.A., and Jureen, R. 2005. Comparison of serotyping, pulsed field gel electrophoresis and amplified fragment length polymorphism for typing of *Streptococcus pneumoniae*. J. Med. Microbiol. 54:467-472.

Sharma, B.S., Jansen, G.B, Karrow, N.A., Kelton, D., and Jiang, Z. 2006. Detection and characterization of amplified fragment length polymorphism markers for clinical mastitis in Canadian holsteins. J. Dairy Sci. 89:3653-3663.

Siemer, B.L., Nielsen, E.M., and On, S.L.W. Identification and molecular epidemiology of *Campylobacter coli* isolates from human gastroenteritis, food, and animal sources by amplified fragment length polymorphism analysis and Penner serotyping. Appl. Environ. Microbiol. 71:1953-1958.

Sim, J., Hood, D., Finnie, L., Wilson, M., Graham, C., Brett, M., and Hudson, J.A. 2002. Series of incidents of *Listeria monocytogenes* non-invasive febrile gastroenteritis involving ready-to-eat meats. Lett. Appl. Microbiol. 35:409-413.

Sims, E.J., Goyal, M., and Arnold, C. 2002. Experimental versus in silico fluorescent amplified fragment length polymorphism analysis of *Mycobacterium tuberculosis*: improved typing with an extended fragment range. J. Clin. Microbiol. 40:4072-4076.

Sinave, C., Le Templier, G., Blouin, D., Léveillé, F., and Deland, É. 2002. Toxic shock syndrome due to *Clostridium sordellii:* a dramatic postpartum and postabortion disease. Clin. Infect. Dis. 35:1441-1443.

Sirisriro, T., Sethabutr, O., Mason, C., Talukder, K.A., and Venkatesan, M.M. 2006. An AFLP-based database of *Shigella flexneri* and *Shigella sonnei* isolates and its use for the identification of untypable *Shigella* strains. J. Microbiol. Meth. 67:487-495.

Slack, A., Symonds, M., Dohnt, M., and Smythe, L. 2006. An improved multiple-locus variable number of tandem repeats analysis for *Leptospira interrogans* serovar Australis: a comparison with fluorescent amplified fragment length polymorphism analysis and its use to redefine the molecular epidemiology of this serovar in Queensland, Australia. J. Med. Microbiol. 55:1549-1557.

Sloos, J.H., Janssen, P., van Boven, C.P.A., and Dijkshoorn, L. 1998. AFLP typing of *Staphylococcus epidermidis* in multiple sequential blood cultures. Res. Microbiol. 149:221-228.

Sloos, J.H., Dijkshoorn, L., Vogel, L., and van Boven, C.P.A. 2000. Performance of phenotypic and genotypic methods to determine the clinical relevance of serial blood isolates of *Staphylococcus epidermidis* in patients with septicemia. J. Clin. Microbiol. 38:2488-2493.

Smith, D, Willshaw, G., Stanley, J., and Arnold, C. 2000. Genotyping of verocytotoxin-producing *Escherichia coli* O157: comparison of isolates of a prevalent phage type by fluorescent amplified-fragment length polymorphism and pulsed-field gel electrophoresis analyses. J. Clin. Microbiol. 38:4616-4620.

Smith, L.D. 1952. The essential characteristics of the species *Clostridium hemolyticum*. J. Bacteriol. 65:222.

Smith-Slatas, C.L., Bourque, M., and Salazar, J.C. 2006. *Clostridium septicum* infections in children: a case report and review of the literature. Pediatrics 117:e796-e805.

Song, Y., Liu, C., Molitoris, D., Tomzynski, T.J., McTeague, M., Read, E., and Finegold, S.M. 2002. Use of 16S-23S rRNA spacer-region (SR)-PCR for identification of intestinal clostridia. System. Appl. Microbiol. 25:528-535.

Songer, J.G. 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9:216-234.

Sood, S., Peters, T., Ward, L.R., and Threlfall, E.J. 2002. Combination of pulsed-field gel electrophoresis (PFGE) and single-enzyme amplified fragment length polymorphism (SAFLP) for differentiation of multiresistant *Salmonella enterica* serotype typhimurium. Clin. Microbiol. Infect. 8:154-161.

Speijer, H., Savelkoul, P.H.M., Bonten, M.J., Stobberingh, E.E., and Tjhie, J.H.T. 1999. Application of different genotyping methods for *Pseudomonas aeruginosa* in a setting of endemicity in an intensive care unit. J. Clin. Microbiol. 37:3654-3661.

Spence, R.P., van der Reijden, T.J.K., Dijkshoorn, L., and Towner, K.J. 2004. Comparison of *Acinetobacter baumannii* isolates from United Kingdom hospitals with predominant Northern European genotypes by amplified-fragment length polymorphism analysis. J. Clin. Microbiol. 42:832-834.

Sperner, B., Eisgrüber, H., and Stolle, A. 1999a. Use of the RAPID ID 32 A system for rapid identification of *Clostridium* species important in food hygiene. Int. J. Food Microbiol. 52:169-180.

Sperner, B., Schalch, B., Eisgrüber, H., and Stolle, A. 1999b. Short protocol for pulsed-field gel electrophoresis of a variety of *Clostridia* species. FEMS Immunol. Med. Mic. 24:287-292.

Stackebrandt, E., and Goebel, B.M. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J. Syst. Bacteriol. 44:846-849.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kämpfer, P., Maiden, M.C.J., Nesme, X., Rosselló-Mora, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A.C., and Whitman, W.B. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 51:1043-1047.

Stolle, A., Sperner, B., Schalch, B., and Eisgrüber, H. 2001. Comparison of protocols for pulsed-field gel electrophoresis of Clostridia. Electrophoresis 22:1585-1589.

Takahashi, H., Handa-Miya, S., Kimura, B., Sato, M., Yokoi, A., Goto, S., Watanabe, I., Koda, T., Hisa, K., and Fujii, T. 2007. Development of multilocus single strand conformation polymorphism (MLSSCP) analysis of virulence genes of *Listeria monocytogenes* and comparison with existing DNA typing methods. Int. J. Food Microbiol. 118:274-284.

Tamada, Y., Nakaoka, Y., Nishimori, K., Doi, A., Kumaki, T., Uemura, N., Tanaka, K., Makino, S.-I., Sameshima, T., Akiba, M., Nakazawa, M., and Uchida, I. 2001. Molecular typing and epidemiological study of *Salmonella enterica* serotype Typhimurium isolates from cattle by fluorescent amplified-fragment length polymorphism fingerprinting and pulsed-field gel electrophoresis. J. Clin. Microbiol. 39:1057-1066.

Taormina, P.J., and Dorsa, W.J. 2004. Growth potential of *Clostridium perfringens* during cooling of cooked meats. J. Food Prot. 67:1537-1547.

Taponen, S., Simojoki, H., Haveri, M., Larsen, H.D., and Pyörälä, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. Vet. Microbiol. 115:199-207.

Taponen, S., Koort, J., Björkroth, J., Saloniemi, H., and Pyörälä, S. 2007. Bovine intramammary infections caused by coagulase-negative staphylococci may persist throughout lactation according to amplified fragment length polymorphism-based analysis. J. Dairy Sci. 90:3301-3307.

Thévenot, D., Delignette-Muller, M.L., Christieans, S., and Vernozy-Rozand, C. 2005. Prevalence of *Listeria monocytogenes* in 13 dried sausage processing plants and their products. Int. J. Food Microbiol. 102:85-94.

Thévenot, D., Delignette-Muller, M.-L., Christieans, S., Leroy, S., Kodjo, A., and Vernozy-Rozand, C. 2006a. Serological and molecular ecology of *Listeria monocytogenes* isolates collected from 13 French pork meat salting-curing plants and their products. Int. J. Food Microbiol. 112:153-161.

Thévenot, D., Dernburg, A., and Vernozy-Rozand, C. 2006b. An updated review of *Listeria monocytogenes* in the pork meat industry and its products. J. Appl. Microbiol. 101:7-17.

Thimothe, J., Nightingale, K.K., Gall, K., Scott, V.N., and Wiedmann, M. 2004. Tracking of *Listeria monocytogenes* in smoked fish processing plants. J. Food Prot. 67:328-341.

Thompson, F.L., Hoste, B., Vandemeulebroecke, K., and Swings, J. 2001. Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. System. Appl. Microbiol. 24:520-538.

Thompson, F.L., Thompson, C.C., Vicente, A.C.P., Theophilo, G.N.D., Hofer, E., and Swings, J. 2003. Genomic diversity of clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991 and 2001 as revealed by fluorescent amplified fragment length polymorphism analysis. J. Clin. Microbiol. 41:1946-1950.

Ticknor, L.W., Kolstø, A.-B., Hill, K.K., Keim, P., Laker, M.T., Tonks, M., and Jackson, P.J. 2001. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. Appl. Environ. Microbiol. 67:4863-4873.

Torpdahl, M., Skov, M.N., Sandvang, D., and Baggesen, D.L. 2005. Genotypic characterization of *Salmonella* by multilocus sequence typing, pulsed-field gel electrophoresis and amplified fragment length polymorphism. J. Microbiol. Meth. 63:173-184.

Torriani, S., Clementi, F., Vancanneyt, M., Hoste, B., Dellaglio, F., and Kersters, K. 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* species by RAPD-PCR and AFLP. System. Appl. Microbiol. 24:554-560.

Unnerstad, H., Bannerman, E., Bille, J., Danielsson-Tham, M.-L., Waak, E., and Tham, W. 1996. Prolonged contamination of a dairy with *Listeria monocytogenes*. Neth. Milk Dairy J. 50:493-499.

Uyttendaele, M., De Troy, P., and Debevere, J. 1999. Incidence of *Listeria monocytogenes* in different types of meat products on the Belgian retail market. Int. J. Food Microbiol. 53:75-80.

Valsangiacomo, C., Baggi, F., Gaia, V., Balmelli, T., Peduzzi, R., and Piffaretti, J.-C. 1995. Use of amplified fragment length polymorphism in molecular typing of *Legionella pneumophila* and application to epidemiological studies. J. Clin. Microbiol. 33:1716-1719.

Van Andel, R.A., Franklin, C.L., Besch-Williford, C.L., Hook, R.R., and Riley, L.K. 2000. Prolonged perturbations of tumour necrosis factor-α and interferon-γ in mice inoculated with *Clostridium piliforme*. J. Med. Microbiol. 49:557-563.

Van Belkum, A., Struelens, M., De Visser, A., Verbrugh, H., and Tibayrenc, M. 2001. Role of genomic typing in taxonomy, evolutionary genetics, and microbial epidemiology. Clin. Microbiol. Rev. 14:547-560.

Van Bergen, M.A.P, Dingle, K.E., Maiden, M.C.J., Newell, D.G., van der Graaf-Van Bloois, L., van Putten, J.P.M., and Wagenaar, J.A. 2005a. Clonal nature of *Campylobacter fetus* as defined by multilocus sequence typing. J. Clin. Microbiol. 43:5888-5898.

Van Bergen, M.A.P., Simons, G., van der Graaf-van Bloois, L., van Putten, J.P.M., Rombout, J., Wesley, I., and Wagenaar, J.A. 2005b. Amplified fragment length polymorphism based identification of genetic markers and novel PCR assay for differentiation of *Campylobacter fetus* subspecies. J. Med. Microbiol. 54:1217-1224.

Van den Berg, R.J., Class, E.C.J., Oyib, D.H., Klaassen, C.H.W., Dijkshoorn, L., Brazier, J.S., and Kuijper, E.J. 2004. Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. J. Clin. Microbiol. 42:1035-1041.

Van den Braak, N., Simons, G., Gorkink, R., Reijans, M., Eadie K., Kremers, K., van Soolingen, D., Savelkoul, P., Verbrugh, H., and van Belkum, A. 2004. A new high-throughput AFLP approach for identification of new genetic polymorphism in the genome of the clonal microorganism *Mycobacterium tuberculosis*. J. Microbiol. Meth. 56:49-62.

Van der Wurff, A.W.G., Chan, Y.L., van Straalen, N.M., and Schouten, J. 2000. TE-AFLP: combining rapidity and robustness in DNA fingerprinting. Nucleic Acids Res. 28:e105.

Van der Zee, A., Steer, N., Thijssen, E., Nelson, J., van't Veen, A., and Buiting, A. 2003. Use of multienzyme multiplex PCR amplified fragment length polymorphism typing in analysis of outbreaks of multiresistant *Klebsiella pneumoniae* in an intensive care unit. J. Clin. Microbiol. 41:798-802.

Van der Zwet, W.C., Parlevliet, G.A. Savelkoul, P.H.M., Stoof, J., Kaiser, A.M., Koeleman, J.G.M., and Vandenbroucke-Grauls, C.M.J.E. 1999. Nosocomial outbreak of gentamicin-resistant *Klebsiella pneumoniae* in a neonatal intensive care unit controlled by a change in antibiotic policy. J. Hosp. Infect. 42:295-302.

Van der Zwet, W.C., Parlevliet, G.A., Savelkoul, P.H., Stoof, J., Kaiser, A.M., van Furth, A.M., and Vandenbroucke-Grauls, C.M. 2000. Outbreak of *Bacillus cereus* infections in a neonatal intensive care unit traced to balloons used in manual ventilation. J. Clin. Microbiol. 38:4131-4136.

Van Eldere, J. Janssen, P., Hoefnagels-Schuermans, A., Van Lierde, S., and Peetermans, W.E. 1999. Amplified-fragment length polymorphism analysis versus macro-restriction fragment analysis for molecular typing of *Streptococcus pneumoniae* isolates. J. Clin. Microbiol. 37:2053-2057.

Vancanneyt, M., Lombardi, A., Andrighetto, C., Knijff, E., Torriani, S., Björkroth, K.J., Franz, C.M.A.P., Foulquié Moreno, M.R., Revets, H., De Vuyst, L., Swings, J., Kersters, K., Dellaglio, F., and Holzapfel, W.H. 2002. Intraspecies genomic groups in *Enterococcus faecium* and their correlation with origin and pathogenicity. Appl. Environ. Microbiol. 68:1381-1391.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60:407-438.

Vaz-Velho, M., Duarte, G., McLauchlin, J., and Gibbs, P. 2001. Characterization of *Listeria monocytogenes* isolated from production lines of fresh and cold-smoked fish. J. Appl. Microbiol. 91:556-562.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., and Kreft, J. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14:584-640.

Velappan, N., Snodgrass, J.L., Hakovirta, J.R., Marrone, B.L., and Burde, S. 2001. Rapid identification of pathogenic bacteria by single-enzyme amplified fragment length polymorphism analysis. Diagn. Micr. Infec. Dis. 39:77-83.

Vijayachari, P., Ahmed, N., Sugunan, A.P., Ghousunnissa, S., Rao, K.R., Hasnain, S.E., and Sehgal, S.C. 2004. Use of fluorescent amplified fragment length polymorphism for molecular epidemiology of leptospirosis in India. J. Clin. Microbiol. 42:3575-3580.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.

Wagenaar, J.A., van Bergen, M.A.P., Newell, D.G., Grogono-Thomas, R., and Duim, B. 2001. Comparative study using amplified fragment length polymorphism fingerprinting, PCR genotyping, and phenotyping to differentiate *Campylobacter fetus* strains isolated from animals. J. Clin. Microbiol. 39:2283-2286.

Wagner, M., Eliskases-Lechner, F., Rieck, P., Hein, I., and Allerberger, F. 2006. Characterization of *Listeria monocytogenes* isolates from 50 small-scale Austrian cheese factories. J. Food Prot. 69:1297-1303.

Waldenström, J., On, S.L.W., Ottvall, R., Hasselquist, D., and Olsen, B. 2007. Species diversity of campylobacteria in a wild bird community in Sweden. J. Appl. Microbiol. 102:424-432.

Wang, X., Maegawa, T., Karasawa, T., Kozaki, S., Tsukamoto, K., Gyobu, Y., Yamakawa, K., Oguma, K., Sakaguchi, Y., and Nakamura, S. 2000. Genetic analysis of type E botulinum toxin-producing *Clostridium butyricum* strains. Appl. Environ. Microbiol. 66:4992-4997.

Wang, Z.Y., Tsoi, K.H., and Chu, K.H. 2004. Applications of AFLP technology in genetic and phylogenetic analysis of penaeid shrimp. Biochem. Syst. Ecol. 32:399-407.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C, Murray, R.G.E., Stackebrandt, E., Starr, M.P., and Trüper, H.G. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463-464.

Werner, G., Willems, R.J.L., Hildebrandt, B., Klare, I., and Witte, W. 2003. Influence of transferable genetic determinants on the outcome of typing methods commonly used for *Enterococcus faecium*. J. Clin. Microbiol. 41:1499-1506.

Whatmore, A.M., Murphy, T.J., Shankster, S., Young, E., Cutler, S.J., and Macmillan, A.P. 2005. Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. J. Clin. Microbiol. 43:761-769.

Wiedmann, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L., and Batt, C.A. 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infect. Immun. 65:2707-2716.

Wieland, B., Regula, G., Danuser, J., Wittwer, M., Burnens, A.P., Wassenaar, T.M., and Stärk, K.D.C. 2005. *Campylobacter* spp. in dogs and cats in Switzerland: risk factor analysis and molecular characterization with AFLP. J. Vet. Med. 51:183-189.

Wieland, B., Sandberg, M., Johannessen, G.S., Bohlin, J., Hofshagen, M., and Cudjoe, K.S. 2006. Genetic variability of *Campylobacter jejuni* isolated from fresh and frozen broiler carcasses. J. Appl. Microbiol. 101:1027-1032.

Wilkins, T.D., and Lyerly, D.M. 2003. *Clostridium difficile* testing: after 20 years, still challenging. J. Clin. Microbiol. 41:531-534.

Willems, A., Doignon-Bourcier, F., Coopman, R., Hoste, B., De Lajudie, P., and Gillis, M. 2000a. AFLP fingerprint analysis of *Bradyrhizobium* strains isolated from *Faidherbia albida* and *Aeschynomene* species. System. Appl. Microbiol. 23:137-147.

Willems, R.J.L., Top, J., van den Braak, N., van Belkum, A., Endtz, H., Mevius, D., Stobberingh, E., van den Bogaard, A., and van Embden, J.D.A. 2000b. Host specificity of vancomycin-resistant *Enterococcus faecium*. J. Infect. Dis. 182:816-823.

Willems, R.J.L., Homan, W., Top, J., van Santen-Verheuvel, M., Tribe, D., Manzioros, X., Gaillard, C., Vandenbroucke-Grauls, C.M.J.E., Mascini, E.M., van Kregten, E., van Embden, J.D.A., and Bonten, M.J.M. 2001. Variant *esp* gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. Lancet 357:853-855.

Wittwer, M., Keller, J., Wassenaar, T.M., Stephan, R., Howald, D., Regula, G., and Bissig-Choisat, B. 2005. Genetic diversity and antibiotic resistance patterns in a *Campylobacter* population isolated from poultry farms in Switzerland. Appl. Environ. Microbiol. 71:2840-2847.

Wong, S., Pabbaraju, K., Burk, V.F., Broukhanski, G.C., Fox, J., Louie, T., Mah, M.W., Bernard, K., and Tilley, P.A.G. 2006. Use of sequence-based typing for investigation of a case of nosocomial legionellosis. J. Med. Microbiol. 55:1707-1710.

Woo, P.C.Y., Ng, K.H.L., Lau, S.K.P., Yip, K.T., Fung, A.M.Y., Leung, K.W., Tam, D.M.W., Que, T.L., and Yuen, K.Y. 2003. Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. J. Clin. Microbiol. 41:1996-2001.

Woo, P.C.Y., Lau, S.K.P., Woo, G.K.S., Fung, A.M.Y., Yiu, V.P.Y., and Yuen, K.Y. 2004. Bacteremia due to *Clostridium hathewayi* in a patient with acute appendicitis. J. Clin. Microbiol. 42:5947-5949.

Woo, P.C.Y., Lau, S.K.P., Chan, K.M., Fung, A.M.Y., Tang, B.S.F., and Yuen, K.Y. 2005. Clostridium bacteraemia characterised by 16S ribosomal RNA gene sequencing. J. Clin. Pathol. 58:301-307.

Woo, P.C.Y, Chung, L.M.W., Teng, J.L.L., Tse, H., Pang, S.S.Y., Lau, V.Y.T., Wong, V.W.K., Kam, K.L., Lau, S.K.P., and Yuen, K.Y. 2007. In silico analysis of 16S ribosomal RNA gene sequencing-based methods for identification of medically important anaerobic bacteria. J. Clin. Pathol. 60:576-579.

Wulff, G., Gram, L., Ahrens, P., and Fonnesbech Vogel, B. 2006. One group of genetically similar *Listeria monocytogenes* sgrains frequently dominates and persists in several fish slaughter- and smokehouses. Appl. Environ. Microbiol. 72:4313-4322.

Zabeau, M., and Vos, P. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application, publication number EP 0534858.

Zhang, C., Zhang, M., Ju, J., Nietfeldt, J., Wise, J., Terry, P.M., Olson, M., Kachman, S.D., Wiedmann, M., Samadpour, M., and Benson, A.K. 2003. Genome diversification in phylogenetic lineages I and II of *Listeria monocytogenes*: identification of segments unique to lineage II populations. J. Bacteriol. 185:5573-5584.

Zhao, S., Mitchell, S.E., Meng, J., Kresovich, S., Doyle, M.P., Dean, R.E., Casa, A.M., and Weller, J.W. 2000. Genomic typing of *Escherichia coli* O157:H7 by semi-automated fluorescent AFLP analysis. Microbes Infect. 2:107-113.