Modern Techniques in Detection, Identification and Quantification of Bacteria and Peptides from Foods

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Academic Dissertation in Microbiology

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Front cover: Pictures from Los Alamos, New Mexico and Finnish lakeside joined by artistic view of DNA helix

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I Kim, Y., J. H. Jett, E. J. Larson, **J. R. Penttila**, B. L. Marrone, and R. A. Keller. 1999. Bacterial fingerprinting by flow cytometry: bacterial species discrimination. Cytometry 36:324-332.

II Larson, E. J., **J. R. Hakovirta**, J. H. Jett, S. Burde, R. A. Keller, and B. L. Marrone. 2000. Rapid DNA fingerprinting of pathogens by flow cytometry. Cytometry 41:203-208.

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

IV **Hakovirta, J.**, J. Reunanen, and P. E. J. Saris. 2006. Bioassay for nisin in milk, processed cheese, salad dressings, canned tomatoes, and liquid egg products. Appl. Environ. Microbiol. 72:1001-1005.

V **Hakovirta, J. R.**, D. Hoornstra, M. S. Salkinoja-Salonen, and P. E. J. Saris. 2008. Microflora of customer returned wines – mitochondriotoxin producing *Bacillus simplex* in wine. Submitted to Applied and Environmental Microbiology.

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THE AUTHOR'S CONTRIBUTION

Paper I:
Janetta Hakovirta contributed to the sample preparation of DNA for pulsed-field gel electrophoresis (PFGE) and for flow cytometry. She performed the PFGE and analysis of the data. She contributed to the writing of PFGE portion of the article.

Paper II:
Janetta Hakovirta contributed to the sample preparation for flow cytometry and to the development of the eight-hour sample preparation. She carried out all the pulsed-field gel electrophoresis (PFGE) experiments and the analysis of the data. Also, she contributed to the writing of the PFGE portion of the article.

Paper III:
Janetta Hakovirta contributed to the single-enzyme amplified fragment length polymorphism experiments, analysis of the results and contributed to the writing of the article.

Paper IV:
Janetta Hakovirta performed all experimental work. She interpreted the results and was the writer of the article.

Paper V:
Janetta Hakovirta performed all experimental work, interpreted the results (excluding the riboprinter analysis), and was the writer of the article.

ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ATCC	American Type Culture Collection
bp	Base pair(s)
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FCM	Flow cytometry
GFP	Green fluorescent protein
GFP_{uv}	Ultraviolet variant of green fluorescent protein
kb	Kilobase pair(s)
LANL	Los Alamos National Laboratory
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
rRNA	Ribosomal ribonucleic acid
SE-AFLP	Single-enzyme amplified fragment length polymorphism
sp./spp.	Species
sv.	Serovar
VNC	Viable but nonculturable

ABSTRACT

Standards have been placed to regulate the microbial and preservative contents to assure that foods are safe to the consumer. In a case of a food-related disease outbreak, it is crucial to be able to detect and identify quickly and accurately the cause of the disease. In addition, for every day control of food microbial and preservative contents, the detection methods must be easily performed for numerous food samples.

In this present study, quicker alternative methods were studied for identification of bacteria by DNA fingerprinting. A flow cytometry method was developed as an alternative to pulsed-field gel electrophoresis, the "golden method". DNA fragment sizing by an ultrasensitive flow cytometer was able to discriminate species and strains in a reproducible and comparable manner to pulsed-field gel electrophoresis. This new method was hundreds times faster and 200,000 times more sensitive. Additionally, another DNA fingerprinting identification method was developed based on single-enzyme amplified fragment length polymorphism (SE-AFLP). This method allowed the differentiation of genera, species, and strains of pathogenic bacteria of *Bacilli, Staphylococci, Yersinia,* and *Escherichia coli*. These fingerprinting patterns obtained by SE-AFLP were simpler and easier to analyze than those by the traditional amplified fragment length polymorphism by double enzyme digestion.

Nisin (E234) is added as a preservative to different types of foods, especially dairy products, around the world. Various detection methods exist for nisin, but they lack in sensitivity, speed or specificity. In this present study, a sensitive nisin-induced green fluorescent protein (GFP_{uv}) bioassay was developed using the *Lactococcus lactis* two-component signal system NisRK and the nisin-inducible *nisA* promoter. The bioassay was extremely sensitive with detection limit of 10 pg/ml in culture supernatant. In addition, it was compatible for quantification from various food matrices, such as milk, salad dressings, processed cheese, liquid eggs, and canned tomatoes.

Wine has good antimicrobial properties due to its alcohol concentration, low pH, and organic content and therefore often assumed to be microbially safe to consume. Another aim of this thesis was to study the microbiota of wines returned by customers complaining of food-poisoning symptoms. By partial 16S rRNA gene sequence analysis, ribotyping, and boar spermatozoa motility assay, it was identified that one of the wines contained a *Bacillus simplex* BAC91, which produced a heat-stable substance toxic to the mitochondria of sperm cells. The antibacterial activity of wine was tested on the vegetative cells and spores of *B. simplex* BAC91, *Bacillus cereus* type strain ATCC 14579 and cereulide-producing *Bacillus cereus* F4810/72. Although the vegetative cells and spores of *B. simplex* BAC91 were sensitive to the antimicrobial effects of wine, the spores of *B. cereus* strains ATCC 14579 and F4810/72 stayed viable for at least 4 months. According to these results, *Bacillus* spp., more specifically spores, can be a possible risk to the wine consumer.

TIIVISTELMÄ (ABSTRACT IN FINNISH)

Mikrobien ja säilöntäaineiden määrälle elintarvikkeissa on luotu ja asetettu raja-arvoja, jotta voidaan varmistaa kuluttajien turvallisuus. Erityisesti ruokamyrkytysepidemioissa on oleellista, että taudinaiheuttajat voidaan havaita ja tunnistaa mahdollisimman nopeasti ja tarkasti. Tämän lisäksi päivittäisessä mikrobien ja säilöntäaineiden seurannassa ja kontrolloinnissa käytettävien ilmaisutekniikoiden ja metodologioiden tulee olla helppokäyttöisiä.

Tässä väitöskirjassa tutkittiin vaihtoehtoisia nopeampia mittausmenetelmiä bakteerien tunnistamiseen DNA-sormenjälkien avulla. Työssä kehitettiin virtaussytometriin perustuva menetelmä vaihtoehdoksi pulssikenttägeelielektroforeesille ("the golden method"). DNA-palojen koon mittaaminen erittäin herkällä virtaussytometrillä mahdollisti lajien ja kantojen erottamisen toistettavalla ja vertailtavissa olevalla tavalla pulssikenttägeelielektroforeesiin nähden. Uusi menetelmä oli satoja kertoja nopeampi ja 200,000 kertaa herkempi kuin vertailumenetelmä. Tämän lisäksi kehitettiin myös toinen DNA-sormenjälkitunnistusmenetelmä. Menetelmässä bakteerin DNA:ta pilkotaan yhdellä entsyymillä ja syntyneitä DNA-paloja monistetaan erityisillä alukkeilla, jolloin syntyy kullekin bakteerille tunnusomainen sormenjälkikuvio. Tämä menetelmä mahdollisti patogeenibakteerien: *Bacilli, Staphylococci, Yersinia,* ja *Escherichia coli* sukujen, lajien ja kantojen erottamisen. Uudella menetelmällä sormenjälkikuviot olivat myös yksinkertaisempia ja helpompia analysoida kuin perinteisellä menetelmällä, jossa käytetään kahta entsyymiä.

Nisiiniä (E234) käytetään yleisesti säilöntäaineena erilaisissa elintarvikkeissa ja erityisesti maitotuotteissa ympäri maailmaa. Nisiinin määrittämiseen on olemassa erilaisia mittausmenetelmiä, mutta ne eivät ole yleensä tarpeeksi herkkiä, spesifisiä ja nopeita. Tässä tutkimuksessa kehitettiin testi, jossa näytteen sisältämä nisiini osoitetaan reportterigeenin avulla. Testissä käytettiin *Lactococcus lactis* –kanta, jolla on kromosomissa kaksikomponenttisignaalijärjestelmä NisRK ja plasmidissa *nisA*-promoottori gfp_{uv} reportterigeenin edessä. Nisiini aktivoi *nisA*-promoottorin NisRK-komponentin välituksellä, mikä saa aikaan reportterigeenin ja vihreän fluoresoivan proteiinin (GFP_{uv}) ilmentymisen, joka voidaan mitata. Testi oli erittäin herkkä ja sillä pystyttiin havaitsemaan jopa 10 pg/ml nisiinipitoisuus kasvatusliemessä. Tämän lisäksi se oli sovellettavissa mittauksiin erilaisista elintarvikkeista, kuten maidosta, salaattikastikkeista, prosessoiduista juustoista, nestemäisistä kananmunista ja säilyketomaatista.

Alkoholipitoisuus, alhainen pH-taso ja orgaanisen aineen pitoisuus antavat viinille hyvät antimikrobiset ominaisuudet, minkä vuoksi viiniä pidetään yleisesti mikrobien suhteen turvallisena elintarvikkeena. Tämän väitöskirjan yhtenä tavoitteena oli tutkia asiakkaiden palauttamia viinejä, joiden oletettiin olleen syynä ruokamyrkytysoireisiin. Osittaisen 16S rRNA –geenisekvenssin analysoinnin, ribotyypityksen ja sian siittiöiden liikkuvuuskokeen avulla yhdestä viinistä löydettiin ja tunnistettiin *Bacillus simplex* BAC91 –bakteeri, joka tuotti siittiösolujen mitokondrioihin vaikuttavaa myrkkyä. Viinin

antimikrobinen aktiviteetti mitattiin *B. simplex* BAC91, *Bacillus cereus* ATCC 14579 (tyyppikanta) ja kereulidi-myrkkyä tuottavan *Bacillus cereus* F4810/72 -bakteerien vegetatiivisilla soluilla ja itiöillä. Vaikka *B. simplex* BAC91 -bakteerin vegetatiiviset solut ja itiöt olivat herkkiä viinin antimikrobisille ominaisuuksille, *B. cereus* tyyppikanta ATCC 14579 ja *B. cereus* F4810/72 -bakteereiden itiöt pysyivät elossa ainakin neljän kuukauden ajan. Tulosten perusteella *Bacillus* spp. ja erityisesti niiden itiöt voivat olla mahdollinen riski viinin kuluttajille.

INTRODUCTION

The challenges in food safety have increased due to the development of new products and production methods, globalization of the markets, increase in consumer knowledge, and to the high demand for different types and healthier foods. Consumers have better understanding of the contents of foods and demand for more reliable and quicker processes to assure good quality. They demand for foodstuff that contains less unnatural components, such as food additives and preservatives, but they also want foods that are safe to eat in regards to pathogenic microorganisms. Therefore, the ability to detect, identify, and quantify unwanted microorganisms and other components in food is important to the food industry as well as to the consumer. Furthermore, these identification techniques are crucial in food-related disease outbreaks for epidemiologic investigations.

1. RISKS IN FOODSTUFF

To the consumer, the possible risks in foods are very broad (Edwards et al. 2007). It can be matter that is clearly foreign to the food, such as metal, glass, plastic and wood or it can be food materials that are foreign to specific food products, such as pork mixed in

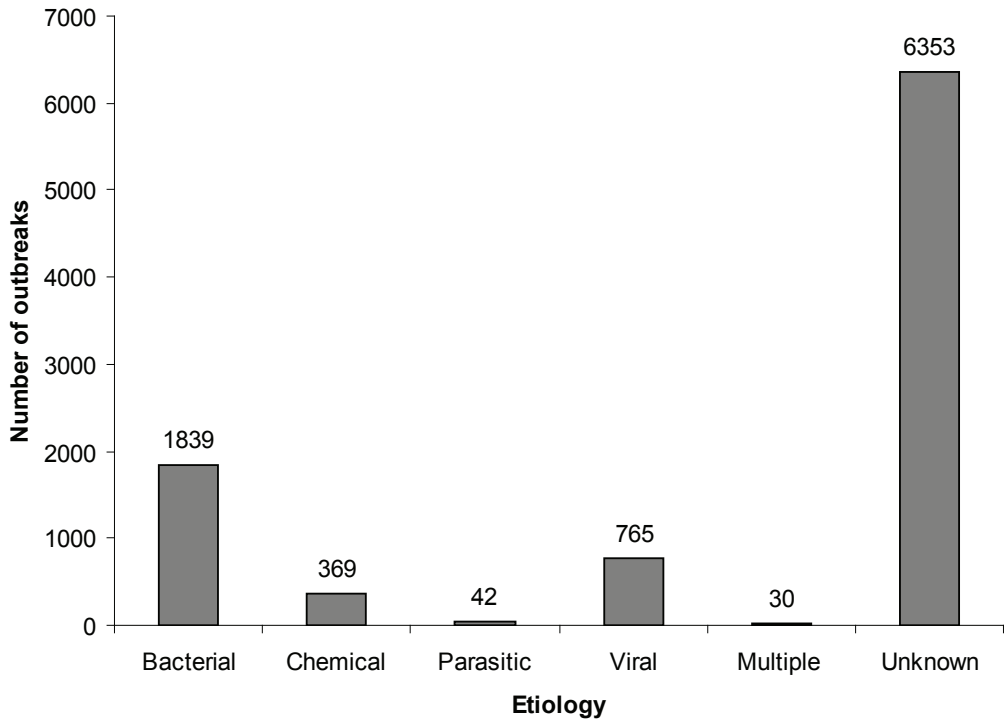

Fig. 1. The etiology of the foodborne illness during 1993-2002 in the United States. (Olsen et al. 2000; Lynch et al. 2006). An outbreak was considered to be two or more cases of a similar illness resulting from the ingestion of a common food.

with other meats or nuts in plain chocolate. It can also be matter that originates from the raw materials, such as bone fragments in meat or fruit stalks in fruit products, but are still unpleasant to the consumer. Another risk to consumers, but perhaps not as visible, is microbes and chemical compounds. It is important to distinguish that these can have negative and positive effects on the food product. Food additives, such as preservatives and colors, are added to foods to improve the organoleptical quality and shelf-life of food products while other chemicals and microbes are required for the preparation of the product. However, if microorganisms contaminate or overgrow in foods, they can cause great havoc to the consumer. Food additives, as well, can be harmful if not added in the proper amounts. Therefore, it is important to constantly control and check the quality of foods.

Foodborne illnesses are a widespread problem throughout the world. These illnesses caused by foodborne microbial pathogens, include fungi, viruses, parasites and bacteria, significantly affect people's health as well as being economically costly. It has been estimated that in the United States approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths will occur annually (Mead et al. 1999), which can cost up to tens of billions of US dollars (Buzby et al. 1996). In developed countries worldwide, this represents that one third of the population are affected by foodborne illnesses each year and it is believed that in developing countries the amount is even higher (Schlundt 2002). Bacteria are the leading causers of illnesses from the other microbial pathogens and threats in food (Fig. 1). The outbreaks reported in the United States during a 10-year period indicate that bacteria were the most common cause of the outbreaks.

In 2006, forty-six food-related outbreaks were reported in Finland and the bacterial causes of these outbreaks were identified as *Bacillus cereus, Bacillus licheniformis, Campylobacter jejuni, Clostridium botulinum, Listeria monocytogenes, Salmonella enterica* serovar (sv.) Typhimurium FT NST*, Staphylococcus aureus,* and *Yersinia pseudotuberculosis* (Evira 2007). Other food-related pathogens are found in the genera *Bacillus*, *Campylobacter*, *Clostridium, Escherichia, Shigella*, *Salmonella*, and *Vibrio* (Woteki and Kineman 2003). It has been approximated that 81% of illnesses and hospitalizations and 64% of deaths related to foodborne illnesses are caused by either unidentified or not detected organisms (Mead et al. 1999).

2. METHODS IN DETECTION AND IDENTIFICATION OF BACTERIA

The traditional way of detecting and identifying bacteria from food, or other samples, is based on culturing, enumeration, and isolation of presumptive colonies for further identification analysis. If necessary, the food sample must be homogenized, concentrated, and/or pre-enriched prior to culturing. Bacterial cells can become injured or viable but nonculturable (VNC) due to the sublethal stressors, such as heat, cold, acid, and osmotic shock, during the food processing steps (Kell et al. 1998). These bacterial cells still pose a threat in the food industry and therefore, methods to improve the detection levels of

these injured cells have been developed. However, even with these methods, all bacterial cells, especially those that are VNC are not detected. The pre-enrichment of the bacteria in a food sample can be performed by a non-selective or selective broth culture (Zhao and Doyle 2001) or by the selective agar overlay technique to resuscitate the injured cells (Hurst 1977; Ray 1986). Another manner in which the detection levels of viable cells can be increased is by concentration of the food sample by filtration or centrifugation prior to plating. More modern methods of concentration or even selecting specific bacteria from heterogenous or polluted samples are by immunomagnetic or by metal hydroxide based separation (Gracias and McKillip 2004).

The pre-treated food sample can then be plated on non-selective, selective and differential media (Gracias and McKillip 2004). Non-selective media or standard methods agar, such as the aerobic plate count, can be used to detect and count the amount of bacteria in the sample. Selective medium contains a compound, such as an antibiotic, bacteriocin, a growth nutrient, which selectively inhibits or ameliorates the growth of specific microorganisms. The third type is a differential medium which contains an indicator, such a chromogenic or fluorogenic substrate, which differentiates bacteria by various chemical reactions carried out during growth. By incorporating fluorogenic or chromogenic enzyme substrates into a selective media, identification of microorganisms can be done directly without further subculturing or biochemical tests. These culture media are based on bacteria producing specific and exact enzymes for substrates. As the enzyme then acts with the substrate, fluorogenic or chromogenic, the bacterial growth will fluoresce or change color, respectively. The developments on chromogenic and fluorogenic culture media for the enumeration and identification of *Escherichia coli*, *Salmonella*, *Clostridium perfringens*, *Bacillus* spp. and *S. aureus* have been reviewed by Manafi (2000).

Even though the culture methods are time consuming and laborious, the isolation and purification of microorganisms allows for further subtyping analysis and for storage in culture collections. The more conventional methods for further subtyping of bacteria include the study of the phenotypic characteristics of the microorganisms. These phenotypic methods include biotyping, serotyping, and phage typing (Arbeit 1995). In biotyping, the biochemical growth requirements, environmental conditions (pH, temperature, antibiotic resistance, bacteriocins susceptibility) and physiological (colony and cell morphology, cell wall composition by microscopy and membrane composition such as by fatty acid analysis) aspects of bacteria (Vandamme et al. 1996) are investigated while serological and phage typing (Towner and Cockayene 1993) concentrate more on the surface structure differences of bacteria. Phages are not only useful in subtyping bacteria, but also in detecting pathogens directly from foods (Hagens and Loessner 2007; Kretzer 2007). However, these phenotypic typing methods are limited since microorganisms are capable of suddenly altering their phenotypic characteristics due to environmental changes or genetic mutations. Therefore, identification by genotypic characteristics has been developed to avoid these problems that can occur with phenotypic methods.

2.1. Nucleic acid amplification methods

2.1.1. Polymerase chain reaction

Polymerase chain reaction (PCR; Saiki et al. 1985) is the amplification of a nucleic acid target sequence. The targeted sequences can be a specific gene, repetitive areas in the sequence or arbitrary sequences. However, when the PCR is based on the amplification of a specific portion of the DNA, the targeted DNA sequence, except for arbitrary PCR, must be known for the synthesis of the oligonucleotides. For foodborne bacterial pathogens, commonly targeted DNA areas are virulence factors, toxins, cellular metabolites, and multicopy ribosomal RNA (rRNA; Olsen et al. 1995; Scheu et al. 1998). The PCR-based techniques have also been developed for screening of genetically modified organisms and their derived materials in foods (Holst-Jensen et al. 2003). Post-PCR detection methods vary from gel electrophoresis, hybridization analysis, and usage of specific nucleic acid probes. In some cases, probes simplify the detection of the PCR product, in the similar way as gel electrophoresis, while in other cases it can further discriminate for only certain bacteria. By using fluorometric or colorimetric labeled probes on PCR products, further detection and specification of species and strains can be performed on membranes or microwells (Mandrell and Wachtel 1999; O´Connor et al. 2000; O´Sullivan et al., 2000; Grennan et al. 2001).

16S rRNA gene is a favorable PCR amplification target by universal or species-strain specific primers for identification and phylogenic purposes since it is universally distributed among bacteria and it contains enough variations amongst strains and species within the DNA sequence (Weisburg et al. 1991). The availability of whole genome to small subunit ribosomal RNA gene sequences, such as 16S rRNA, data is constantly increasing and public-domain databases have been established, such as Ribosomal Data Base Project (http://rdp.cme.msu.edu/) and National Center for Biotechnology Information Blast Library (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). These database libraries can be applied for identification of cultured and uncultured microorganisms from environmental, clinical, and food samples by comparing the 16S rRNA gene sequences in the databases to those of the unknown microorganism (Drancourt et al. 2000). However, 16S rRNA gene sequence analyses have shown limited variability within strains of a bacteria species (Bottger 1989; Woese 1987; Olsen and Woese 1993). This is especially evident amongst homogenous groups, such as the cereulide-producing *B. cereus*. For example, Apetroaie et al. (2005) showed that the 16S rRNA gene sequences of thirteen cereulide-producing *B. cereus* strains were identical to each other as well as to the 16S rRNA gene sequences of *Bacillus anthracis* strains Ames, Sterne, and NC 08234-02. Therefore, 16S rRNA gene sequence analysis for homology is not always capable to completely identify an unknown organism.

2.1.2. Variations of PCR

Different variations of PCR have been developed in order to fulfill simultaneous detection of multiple bacteria, quantification, and differentiation of viable bacterial cells from foods. Simultaneous bacterial detection can be performed by multiplex PCR, which uses several different primers targeted for specific genes of each bacterial strain (Yaron and Matthews 2002; Touron et al. 2005). Many multiplex PCR systems have

been developed for differentiation of multiple species belonging to single genera and for differentiation of mixed bacterial pathogens (Settanni and Corsetti 2007). Conventional PCR is not able to indicate if the bacterial cells are viable or dead and therefore reverse transcriptase PCR was developed for the specifically detecting viable cells. This method is based on the reverse transcriptase enzyme that is able to use messenger RNA as a template for synthesizing single-stranded DNA in the 5' to 3' direction (Lazcka et al. 2007; Rodríguez-Lázarro et al. 2007). The technique is sensitive and requires no pre-enrichment steps, which decreases the time of analysis (Deishing and Thompson 2004). Another advantage of the reverse transcriptase PCR is the detection of VNC cells that are not detected with culturing. For the quantitative detection of bacteria in a sample, quantitative PCR can be used (Monis and Giglio 2006). This technology is based on the monitoring of formation of PCR product simultaneously as the reaction occurs by using fluorescent probes or dyes that are sequence specific or nonspecific. Molecular beacons are an example of a sequence specific fluorescent probe which undergoes a fluorogenic conformational change when hybridizing to their target (Tyagi and Kramer 1996). SYBER Green I and SYBER gold are nonspecific dyes that bind to the double-stranded DNA of the PCR product (Glynn et al. 2006). Only fluorescent probes labeled with different reporter dyes can be used to detect multiple amplicons within the same reaction mixture while double-stranded DNA dyes are limited to a single product per reaction (Robertson and Nicholson 2005).

2.1.3. Limitations of nucleic acid amplification methods

The acceptance and application of nucleic acid amplification methods in routine detection of foodborne pathogens has been limited due to the standardization and validation of PCR protocols. The protocols need to be synchronized between laboratories, so that the PCR results are reliable and reproducible when performed in different locations or times (Malorny et al. 2003). However, this is problematic because many of the aspects that can affect PCR are difficult to control. These are the quality of the DNA template, the environment (humidity, chemical and microbiological cleanliness, temperature), the equipments, personal practice and the reaction conditions and the reaction materials (Malorny et al. 2003). In addition, food itself is a difficult matrix since it can contain substances that affect the PCR reaction. Food can contain substances that can degrade the target nucleic acid sequence or inhibits the enzyme activity in the PCR, which can give false negative results (Glynn et al. 2006). Also PCR does not confirm the presence of toxins in the food, but only the genetic potential of a microorganism to produce them (de Boer and Beumer 1999). Although the technique of PCR is simple and quick, the technique still needs improvement for bacterial investigations of different foods and for standardized protocols in public health laboratories.

2.2. Restriction endonuclease analysis

By using restriction enzymes, the nucleic acid sequence differences in the whole genome DNA or plasmid can be studied without knowing the actual DNA sequence of the microorganism (Towner and Cockayne 1993). Many different types of restriction enzymes are available and they cleave DNA only at specific recognition sites resulting in a unique restriction pattern of DNA fragments, called a fingerprint. Bacteria can be typed and identified by comparing the DNA fingerprinting patterns.

Plasmids are separate genetic elements that can replicate independently from the main chromosome. They usually encode useful additional properties of the cells, as antibiotic or bacteriocin resistance, metabolic activity, but they do not code the necessary genes for survival, the housekeeping genes of the cell. Plasmids are found in a vast majority of the bacterial genera. However, plasmids are not stable or the strain might lack the plasmid and therefore using plasmids for DNA fingerprinting pattern typing is not reliable (Towner and Cockayne 1993; Arbeit 1995; van Belkum et al. 2007). However, using the complete chromosome for DNA fingerprinting analysis will result in hundreds of fragments resulting in a complex fingerprint pattern that is difficult to analyze. The number and sizes of the restriction fragments generated by digestion are influenced by both the recognition sequence of the enzyme and the nucleic acid composition of the DNA. By using low frequently cutting enzymes or by combining the enzyme digestion technique with other typing methods, the fragment amounts can be decreased and the analysis simplified.

2.2.1. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is based on the digestion of chromosomal DNA by rare cutting enzymes. By using such enzymes, the total amount of DNA fragments is minimized. However, the resulting DNA fragments are large and therefore cannot be separated by conventional gel electrophoresis where gel matrix sieves the different sized DNA molecules under unidirectional electric field. Schwartz and Cantor (1984) were the first to describe a new type of gel electrophoresis method that they used to produce a molecular karyotype from the chromosomal DNA of yeast *Saccharomyces cerevisiae*. This new method was capable of separating large DNA molecules up to 2000 kb by applying alternately pulsed electric fields that were perpendicular to each other and of which one was inhomogeneous. Different variations in the instrumentation have been developed since 1984 to improve the resolution and diminish distortion of the DNA fragments (Towner and Cockayne 1993). However, the basic principle of PFGE is using successive alternating electric fields which allow the DNA molecules to change continuously their direction of migration. The separation is not due to sieving, as in conventional agarose gel electrophoresis, but on the size of the DNA molecule. Larger DNA molecules change directions more slowly than smaller molecules. The large DNA molecule coils open and elongate parallel to an electric field in which manner it can enter a pore opening in the agarose. When the electric field stops and a new electric field is applied perpendicular to the opened DNA, the DNA molecule has to re-orient itself to enter a new opening. If the field direction is switched too slowly, the DNA molecule cannot reorient itself resulting in a situation as in conventional electrophoresis. The pulse time (ramping) and electron force (gradient) is constantly increased to achieve better separation of all different sizes of DNA fragments (Towner and Cockayene 1993). By having size standards, the sizes of the DNA fragments can be analyzed and the fingerprint patterns can be compared to other bacterial fingerprints. PFGE is capable of separating DNA molecules from 50 kb – 12 Mb (Towner and Cockayen 1993).

Since large DNA molecules break easily even from small amounts of shear, their isolation is difficult by the common isolation methods performed in solution. Schwartz

and Canton (1984) also described an alternative isolation method which was based on embedding cells into agarose to minimize the shearing of DNA during the isolation steps. Different variations of this procedure (Gautom 1997; Chang and Chui 1998; Kim et al. 1999; Larson et al. 2000) have been described as well, but the overall concept is the same (Fig. 2).

PFGE has proven to be highly discriminatory and superior to many of the other typing methods (Arbeit 1995). It is capable of differentiation between species and strains and therefore it has been investigated for the use in epidemiological studies, such as with *Campylobacter coli* (Yan et al. 1991), *C. jejuni* (Yan et al. 1991), *L. monocytogenes* (Brosch et al. 1991), and *S. aureus* (Schlichting et al. 1993). Zhong et al. (2007) developed a PFGE method for discriminating *B. anthracis* from the closely related species of *B. cereus* and *Bacillus thuringiensis* by slighty modifying the DNA isolation method to improve lysis and enzyme digestion with *Not*I of these spore-forming species. Although the 25 different strains of *B. anthracis* DNA fragment patterns were different from the strains of *B. cereus* and *B. thuringiensis*, the fingerprint patterns of the *B. anthracis* strains were 94% similar to each other. However, the method was not

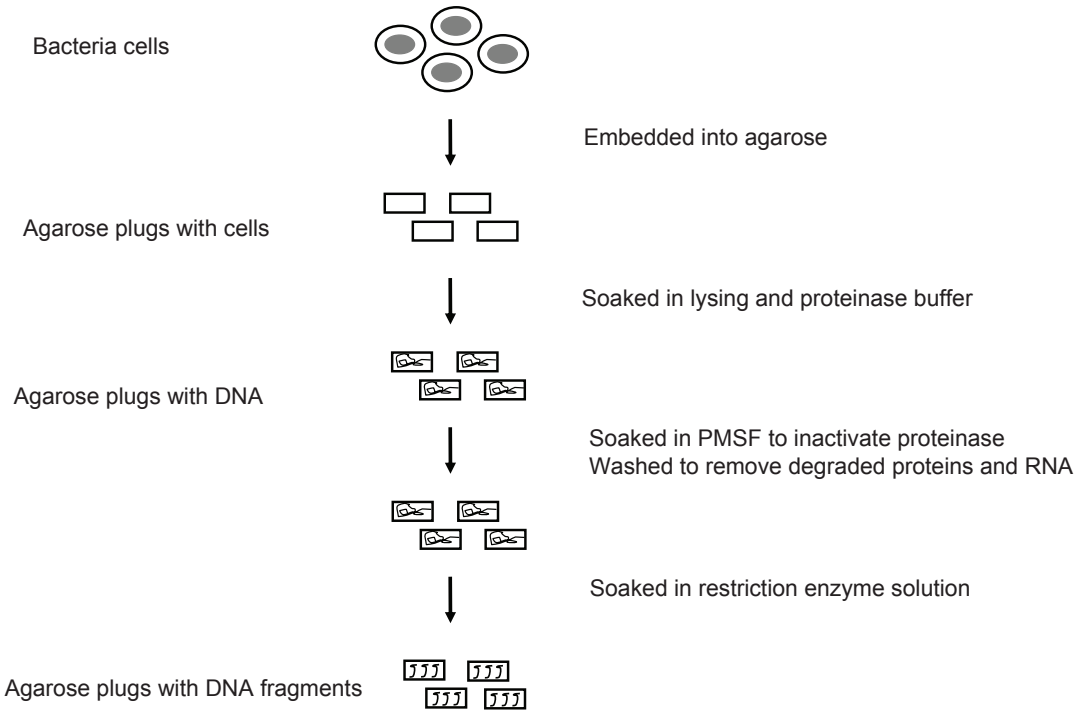

Fig. 2. Schematic representation of the agarose plug procedure for DNA isolation and restriction enzyme digestion (Kim et al. 1999). Bacterial cells are harvested and embedded into low temperature agarose to form plugs. The cell walls are lysed and proteins and RNA degraded enzymatically to access the DNA in the cells. The proteinase is inactivated by phenylmethylsulfonyl fluoride and the degraded protein and RNA removed by multiple buffer washes. The plugs containing clean DNA are then digested with a restriction enzyme.

able to segregate *B. cereus* and *B. thuringiensis* from each other, which has also been reported earlier with PFGE (Carlson et al. 1994). Other methods, such as amplified fragment length polymorphism (Hill et al. 2004) and multilocus enzyme electrophoresis of housekeeping genes and their sequence analysis (Helgason et al. 2000), have not been able to distinguish between these species. In addition, it can differentiate between methicillin-resistant *S. aureus* strains (Prevost et al. 1992), vancomycin- and ampicillin-resistant *Enteroccous faecium* (Price et al. 2002; Jureen et al. 2004) while ribotyping was not as effective in differentiating these strains. In 1996, PFGE became the standard procedure for bacterial foodborne disease outbreak analysis (Swaminathan et al. 2001) due to its discriminatory capabilities (Gerner-Smidt et al. 2006). Uniform guidelines for performing PFGE and interpretation of the data have been set up to confirm the reproducibility amongst laboratories (Tenover et al. 1995). Therefore, it is considered the "golden standard" for molecular-based studies. It has become the preferred subtyping method for networks that have been created within the United States (PulseNet) and Europe (PulseNet Europe) for surveillance and for collection of PFGE fingerprints of bacterium related to foodborne infections (Swaminathan et al. 2001; Rodríguez-Lázaro et al. 2007). Currently, PulseNet USA has standardized PFGE protocols for Shiga toxigenic *E. coli* O157*, S. enterica, Shigella* spp., *L. monocytogenes,* thermotolerant *Campylobacter* spp., and *V. cholerae* and *S. enterica* sv. Braenderup strain H9812 digested with *Xba*I as the universal standard (Gerner-Smidt et al. 2006).

2.2.2. Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) is a genome fingerprinting technique based on the PCR amplification of only certain fragments that have been the result of restriction digestion of the whole genome (Vos et al. 1995; Lin et al., 1996; Olive and Bean 1999). The basic procedure includes the enzyme digestion by two restriction enzymes that yield DNA fragments with two different types of sticky ends. To these ends, adapters are ligated to form templates for the PCR. The selective amplification reaction is performed by using two different primers containing the same sequence as the adapters, but extended to include one or more selective bases next to the restriction site of the primer. Only fragments that are completely a match are amplified. This technique results in about 30 to 40 DNA fragments, some of which are species specific while others are strain specific (Janssen et al. 1996; Jackson et al. 1999; Koeleman et al. 1998; Jureen et al. 2004; Melles et al. 2007).

2.2.3. Restriction fragment length polymorphism

Restriction fragment length polymorphism combines restriction enzyme digestion and Southern blot hybridization (Olive and Bean 1999). The chromosomal DNA is enzymatically digested resulting in DNA fragments that are separated by gel electrophoresis. These fragments are the transferred to either a nitrocellulose or nylon membrane. The membrane bound fragments are then hybridized with one or multiple probes that are specific for a certain gene or sequence. The probes can be labeled with detectable moieties, such as radioactive isotopes, enzyme-colorimetric or enzyme-chemiluminescent substrates (Arbeit 1995; Olive and Bean 1999). Due to the species and strain differences in the location of the restriction enzyme sites and with the specificity of the probe, the resulting fingerprint is simplified and therefore, easier to analyze.

The rRNA probe is more applicable for a wide variety of bacteria than other probes that are more species or strain specific (Towner and Cockayne 1993). The use of this probe for characterization is called ribotyping where restriction enzyme digestion and Southern blot hybridization are used together for analysis. Since the ribosomal operons in bacteria are organized into 16S, 23S and 5S rRNA and are often separated by non-coding spacer DNA (Towner and Cockayne 1993), the probe can be either one of the rRNA genes or a mixture or parts of the rRNA genes and the spacer sequences. However, hybridization patterns differ depending on the probe used (Saunders et al. 1990). Labeled probes containing *E. coli* 23S, 16S and 5S rRNA sequences are most often used for ribotyping (Bingen et al. 1994). Ribotyping has been shown to be advantageous in identifying strains, such as *Pseudomonas aeruginosa* isolated from the mucous of a cystic fibrosis patient and *Legionella* spp., which are difficult to type with the classical phenotypic methods (Bingen et al. 1994). From studies with *L. monocytogenes, P. aeruginosa,* and *Salmonella*, ribotyping showed to be a practical and rapid method for screening of large number of isolates, but alone it is not necessarily the best method for discriminating between strains and other typing methods, such as PFGE or serotyping, are required (Grundmann et al. 1995; Bailey et al. 2002; Aarnisalo et al. 2003; Lukinmaa et al 2004).

2.3. Immunological methods

Serological typing is one of the oldest immunological-based techniques, but still an important tool in identifying bacteria that is based on the difference, even within a species, of antigenic determinants expressed on the cell surface (Towner and Cockayne 1993; van Belkum et al. 2007). These surface structures include lipopolysaccharides, capsular polysaccharides, membrane proteins and extracellular organelles, such as flagella and fimbriae that react with antibodies (Arbeit 1995). Serotyping is still important for gram-negative bacteria, such as *Campylobacter, E. coli* and *Salmonella*, and also some gram-positive bacteria, such as those in the genus *Listeria*. (Nachamkin 2001; Jay et al. 2005). The simplest antibody tests are latex agglutination for bacterial cells and reverse passive agglutination for antigens that are soluble, such as toxins (Feng 2001). The main difference is that in latex agglutination bacterial surface antigens bind to beads with antibodies and this causes glumping while for insoluble materials it forms a diffuce lattice. Immunofluorescence is another traditional subtyping technique that is also based on surface-associated antigens that are detected by antibodies directly labeled with fluorescein or a fluorescein-labelled conjugate which is added for visualization of the antigen-antibody binding (Towner and Cockayene 1993). Immunoassays not only exist for surface antigens, but also for detection of metabolites, such as toxins, and assays have been described for botulinum, cholera, *Staphylococcal* enterotoxin, *C. perfringens* enterotoxins, and *B. cereus* enterotoxins (Notermans and Wernars 1991).

More modern versions of immunological methods have been developed, so that larger quantities of samples can be analyzed with less time and effort and using food directly rather than an isolated and purified bacterial isolate, such as with latex agglutination (Gracias and McKillip 2004). At the present, the enzyme-linked immunosorbent assay (ELISA) is the most established immunological technique, from which the indirect or

sandwich-ELISA (Fig. 3) is the more commonly used for pathogen detection (Lazcka et al. 2007).

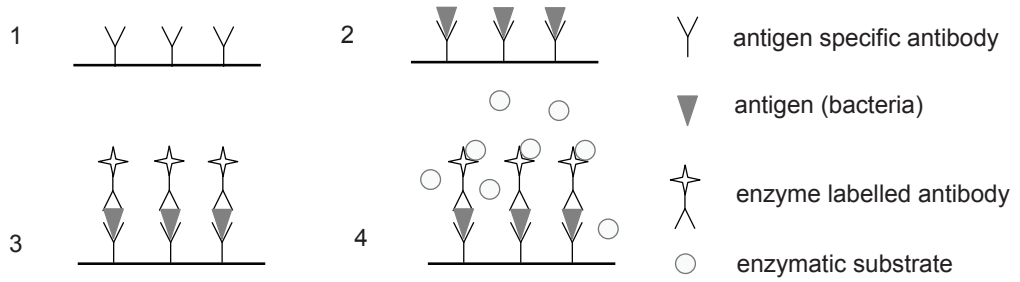

Fig. 3. Schematic presentation of the steps in the indirect enzyme-linked immunosorbent assay: 1) antigen-specific antibody immobilized to a surface; 2) addition of antigen; 3) addition of enzyme labeled antibody; 4) color detection by eye or spectrophotometer due to addition of substrate to enzyme.

ELISA techniques have been developed for the detection of whole cell antigen targets or products for such pathogens as *B. cereus* (Chen et al. 2001), *Campylobacter* spp. (Bolton et al. 2002), *E. coli* (Daly et al. 2002), and *Salmonella* spp. (Peplow et al. 1999) from foods.

2.4. Future methods in detection and identification of bacteria

Constantly more rapid and easier techniques are being developed for simultaneous detection and identification of bacteria from food. These techniques are based on the similar concepts as the previously mentioned phenotypic and genotypic methods, but they include new instrumentation or set-ups for the analysis of the samples. Although flow cytometry, DNA microarrays and biosensors will be discussed in this thesis, other bacterial detection systems have been developed based on instruments that are traditionally used in the field of chemistry, such as reflectance spectroscopy (Rahman et al. 2006), Fourier transform Raman spectroscopy (Yang and Irudayaraj 2003) and mass spectrometry (Mandrell and Wachtel 1999).

2.4.1. Flow cytometry

Although flow cytometry (FCM) was discovered in the late 1960's, its applicability in the field of microbiology has not yet been fully reached. It has been extensively applied to mammalian cells and chromosomes, such as cell cycle analysis and medical diagnostic studies (Steen 2000; Longbardi Givan 2001). However, its potential in microbiology is continuously investigated and therefore it can still be considered a developing technique in field of detection and identification of bacteria.

2.4.1.1. Instrumentation of FCM

Steen (2000) described FCM simply as a fluorescent microscope with cells flowing through the focus. The instrument composes of four main elements: a light source,

fluid lines and controls (fluidics), electronic network, and a computer (Longobardi Givan 2001). Optical lenses shape the laser beam (light source) into a focused light that illuminates the samples one at a time, such as cells. As the cells pass through the illumination spot in the FCM, sheath fluid surrounds them to assure the uniformity of the alignment between the cells and the laser beam. At the analysis point, lenses collect the light signal from the cells by focusing it onto photodetectors (photodiodes and photomultipliers) that convert the light signal to an electrical signal. The electrical signal is then converted from analog-to-digital and analyzed accordingly using standard analysis computer software.

In a FCM (Fig. 4), there are multiple photodetectors that measure simultaneously different aspects from a single cell (Longobardi Givan 2001). The forward-angle photodiode is located directly at the analysis point. An obscuration bar is in front of this detector to only allow the detection of the light that has been bent by passing through the cell. This signal is called forward scatter or forward-angle light scatter which is related to the size or volume and the refractive index of the cell (Davey and Kell 1996). To the right angles of the illuminating beam, three or more photodetectors are located that detect any light that is deflected to the side from the analysis point. Since photodetectors measure all colors of light, filters must be in front of them to specify the light which each one measures. One of the photodetectors registers the illuminating light that has been bounced 90º from the surface of the analyzed cell. This signal is called side scatter light which reflects the granularity of a particle (Davey and Kell 1996). The other photomultipliers are there to detect other colors of light that might be emitted by the cell due to endogenous fluorescent compounds or to staining by fluorescent dyes which allow the study of surface proteins, intracellular proteins and DNA (Nebe-von-Caron et al. 2000; Brehm-Stecher and Johnson 2004).

2.4.1.2. FCM in microbiology

The size of bacteria is one of the obstacles that has slowed down the utilization of FCM in bacteriological investigations. The diameter of a bacterial cell is around one-tenth of that of a mammalian blood cell resulting in a smaller surface area for staining, and the

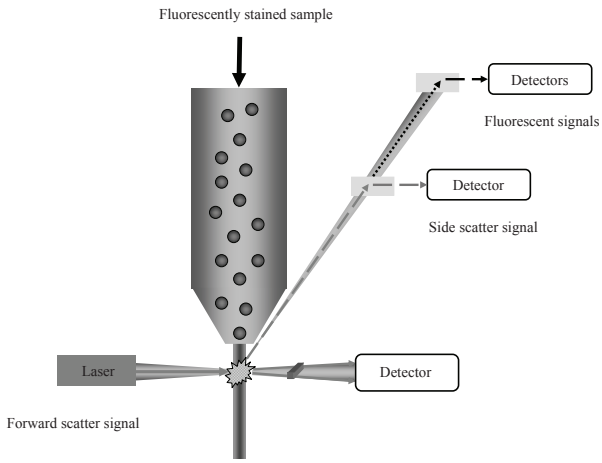

Fig. 4. Principal of flow cytometry. A fluorescently stained sample is illuminated by a laser light. The forward scatter detector is in line with the laser beam (grey, solid arrows). The side scatter detector measures light that is of the same color as the laser beam, but scattered at a 90º angle (grey, dashed arrows). The other detectors detect colors different of the laser beam that might be emitted by the sample (black, dotted arrows).

DNA content of a bacterial cell, such as *E. coli*, is about 10^{-3} times that of diploid human cell (Longbardi Givan 2001). Therefore, the bacterial cells require sensitive instruments and bright fluorescent dyes (Steen 2000). The first microbiologists studied the nucleic acid and protein amounts in bacterial cells during different growth stages by FCM (Bailey et al. 1977; Paau et al. 1977; Steen 2000). With improvements in the sensitivity of FCM and in dyes, FCM studies in bacteriology have become more common. FCM has developed into an intriguing tool for microbiology research due to its capability to simultaneously measure multiple aspects of a homogenous or heterogenous sample, such as cell detection, cell counting, and cellular structure analysis (Brehm-Stecher and Johnson 2004).

Microbial identification by FCM is based on the bacterial properties that are also used in phenotypic and genotypic methods described earlier in the thesis. The size and granularity of the cells is indicated by the light scatter which can be used in differentiating cells, such as yeast from bacterial cells (Malacrinò et al. 2001). The autofluorescent properties of cells, such as the presence of photosynthetic pigments, have been used in identifying and classifying algae (Troussellier et al. 1993). FCM has been utilized in serological discrimination of bacteria, fungi, viruses and parasites (Álvarez-Barrientos et al. 2000). In molecular biology, FCM can be used for DNA fragment analysis of bacteria (Huang et al. 1999) and viruses (Ferris et al. 2005), and detection of clones and mutants by reporters encoded by genes, such as *lux* and *gfp* (Huang et al. 1996; Link et al. 2007). In food microbiology (Ueckert et al. 1995), FCM is advantageous since it can be used in differentiating between dead, viable, and VNC cells by using fluorescent dyes (Breeuwer and Abee 2000) that indicate the membrane integrity, membrane potential, respiration, intracellular pH, and enzyme activity of cells, directly from food, such as milk, juice, wine, vegetable products, and ground beef (Laplace-Builhé et al. 1993; Gunasekera et al. 2000; Malacrinò et al. 2001; Yamaguchi et al. 2003).

2.4.2. DNA microarrays

DNA microarrays consist of a solid surface (glass, silicon, nylon substrates) to which a large number of probes, DNA fragments or oligonucleotides, are immobilized that will hybridize to fluorescently labeled DNA (target) from the sample (Call 2005). The target can be genomic DNA isolated from the sample or an amplified PCR product. Genomic microarrays and oligonucleotide arrays are the two types of DNA microarrays. In genomic DNA microarrays, the probes are complete genes or their fragments from a strain of a microorganism, while in oligonucleotide microarrays the target DNA hybridizes to 18 to 70 nucleotides long oligos. Although both types of microarrays can be used in detection of pathogens, commonly oligonucleotide microarrays are used in detection of either genomic DNA directly or PCR amplified portion of the genomic DNA, such as rRNA genes or virulence genes (Kostrzynska and Bachand 2006). Microarrays have been developed for identification of food-borne bacterial pathogens belonging to *Bacillus* spp., *C. jejuni, E. coli, L. monocytogenes, S. enterica*, *Shigella dysenteriae, Staphylococcus* spp., and *Vibrio* spp. (Call et al. 2003; Chiang et al. 2006; Garaizar et al. 2006; Sergeev et al. 2006; Eom et al. 2007) and for discrimination from multiple different pathogens and their virulence factors (Sergeev et al 2006; Wang et al.

2007b) in case of food outbreaks and biological warfare (Sergeev et al 2004; Wang et al. 2007b).

Due to the fact that multiple genetic properties can be analyzed at the same time and their flexibility in developing arrays that are specific for certain analyses, DNA microarrays make excellent tool in epidemiological studies and food safety control. However, improvements in microarrays are still required for them to become more economical and practical for public health laboratories and food industries (Garaizar et al. 2006; Kostrzynska and Bachand 2006).

2.4.3. Biosensors

According Lazcka et al. (2007), biosensor technology is the fastest growing technology for pathogen detection when compared to PCR, immunology, culture methods and gel electrophoresis. A biosensor consists of a biological material, biologically derived material, or a biomimic that is associated or integrated to a transducer. This transducer can be physiochemical or biological that converts the detected change or presence of a various analyte in the analyzed sample into a measurable signal (Lazcka et al. 2007). These devices can be used to detect analytes, such as carcinogens, pollutants, drugs, pesticides, and pathogens from water, waste, soil and foods (Arora et al. 2006). Various detection methods based on biosensors have been applied in detection of food pathogens. These sensors have been based on DNA, immunology, and phage display peptides (Table 1).

Transducers are based on optical, acoustical, and electrochemical signal detection (Lazcka et al 2007). Optical biosensors measure changes in fluorescence, luminescence, absorbance, or refractive index. Fluorescence techniques are based on direct measurement of fluorescent indicator compounds, or in the case of fluorescence resonance energy transfer biosensors (Baeumner 2003), a donor fluorophore donates energy to an acceptor fluorophore, which then emits light (Ko and Grant 2003). Surface plasmon resonance is based on detecting changes in refractive index caused by structural alterations of a thin film metal surface, such as gold. (Cooper 2003). Acoustic sensors measure changes in resonance frequency, due to a mass change of a bio-molecular surface, such as piezoelectric crystals, e.g. quartz (O'Sullivan and Guilbault 1999). Another sensor type is electrochemical biosensors designed to measure changes as current and potential at the sensor/sample matrix interface (Laczka et al. 2003). These sensors are classified in respect to what they measure: amperometric (current), potentiometric (potential), and impedimetric (impedance).

Whole cells and higher organisms (plants, algae, nematodes, animal tissues) have been used as detectors in biosensors (Baeumner 2003). In this thesis, emphasis is placed on bacterial biosensors. Viable microbes produce metabolites, such as carbon dioxide, ammonia, acids, or they are bioluminescent as exemplified by *Vibrio fischeri*, which can be used to monitor viability (D'Souza 2001). Many microbial biosensors are based on light emission from luminescent or fluorescent bacteria that are genetically engineered to express fluorescent or luminescent proteins, such as green fluorescent protein (GFP)

or luciferase protein (D'Souza 2001; Baeumner 2003). So far, microbial biosensors and bioassays have been applied more prevalently in the detection of food additives and food contaminants than in direct monitoring of food pathogens themselves.

Table 1. Various biosensor methods for detection of food pathogens and other food-related compounds.

Detection technique	**Organisms /Compounds detected**	
DNA based biosensors	Pathogens	*Bacillus anthracis, Escherichia coli, Listeria monocytogenes*
	Compounds	Aflatoxins, PCB, pesticides
Enzyme based biosensors	Pathogens	*E. coli* O157:H7, *Salmonella enterica* sv. Typhimurium
	Compounds	Pesticides, antibiotics (milk), benzoic acids (soda drinks), L-lactase[1] (tomato paste), biogenic amines[1] (sauerkraut)
Antibodies and receptors based biosensors[2]	Pathogens	*Bacillus cereus, Campylobacter* spp., *E. coli, L. monocytogenes, S. enterica* sv. Enteritidis, *S. enterica* sv. Typhimurium, *Staphylococcus aureus, Yersinia pestis*
	Compounds	Pesticides, antibiotics (milk), organic solvents, alfatoxins, staphylococcal enterotoxin B

The methods were compiled from Arora et al. 2006 and Bauemner 2003.
[1] compounds for indicating freshness of food
[2] combines immunological and phage display peptide biosensors

AIMS OF THE STUDY

Food safety is important to regulators and consumers. Food poisoning cases are still common, even if the knowledge about food preservation and good hygiene has improved throughout the world. In a case of a food and water epidemics or in biological warfare, the organism(s) must be detected and identified rapidly to stop the spreading of the illness. Additionally, food preservatives and food additives are strictly regulated and controlled, so rapid methods are also required for their detection and quantification. The aims of this thesis were the following:

1. Develop a faster method to detect and identify bacteria at species and strain level by flow cytometry.
2. Develop a SE-AFLP method for genus, species, and strain differentiation among bacteria of *Bacillus* spp.*, E. coli, Staphylococcus* spp. and *Yersinia* spp.
3. Develop an extremely sensitive bioassay to detect and quantify nisin, an antimicrobial food preservative, from different food matrices.
4. Apply 16S rRNA gene sequence analysis to study the microflora, including potential pathogens, of customer returned wines.

MATERIALS AND METHODS

The bacterial strains used in this study are shown in Table 2 and Table 3, plasmids in Table 4, restriction enzymes in Table 5, and primers in Table 6. The methods used in this study are indicated in Table 7, but are described in more detail in the Materials and Methods sections of the Papers I-V.

Table 2. Bacterial strains used in this study.

Bacterial strain	Source	Used in
Bacillus cereus 4342	ATCC, Rockford, MD, USA	III
B. cereus 7064	ATCC, Rockford, MD, USA	III
B. cereus 11778	ATCC, Rockford, MD, USA	III
B. cereus 14579	ATCC, Rockford, MD, USA	III, V
B. cereus 33018	ATCC, Rockford, MD, USA	III
B. cereus F4810/72	HAMBI, University of Helsinki, Finland	V
Bacillus globigii	Aberdeen Proving Ground, MD, USA	I
Bacillus mycoides 6463	ATCC, Rockford, MD, USA	III
B. mycoides 19647	ATCC, Rockford, MD, USA	III
B. mycoides 23258	ATCC, Rockford, MD, USA	III
Bacillus simplex BAC91	This study	V
Escherichia coli 11775	ATCC, Rockford, MD, USA	II, III
E. coli 15597	ATCC, Rockford, MD, USA	I
E. coli 25254	ATCC, Rockford, MD, USA	III
E. coli 25922	ATCC, Rockford, MD, USA	I, II
E. coli 27622	ATCC, Rockford, MD, USA	II, III
E. coli 33694	ATCC, Rockford, MD, USA	II, III
E. coli 35326	ATCC, Rockford, MD, USA	III
E. coli 43888	ATCC, Rockford, MD, USA	III
E. coli 43893	ATCC, Rockford, MD, USA	III
E. coli 43895	ATCC, Rockford, MD, USA	III
E. coli MG 1655	University of Wisconsin-Madison, WI, USA/ATCC, Rockford, MD, USA	II, III
Erwinia herbicola	Aberdeen Proving Ground, MD, USA	I
Staphylococcus aureus 10832	ATCC, Rockford, MD, USA	II, III
S. aureus 12600	ATCC, Rockford, MD, USA	II, III
S. aureus 13301	ATCC, Rockford, MD, USA	II, III
S. aureus 25923	ATCC, Rockford, MD, USA	II, III
S. aureus 29213	ATCC, Rockford, MD, USA	II, III
S. aureus 29996	ATCC, Rockford, MD, USA	II, III
Lactococcus lactis NZ9000	NIZO/Kuipers et al. 1998	IV
L. lactis LAC275	This study	IV

ATCC = American Type Culture Collection

Table 3. Only DNA from these bacterial strains were used.

Bacterial strain	Source	Used in
Bacillus anthracis Vollum	LANL, NM, USA	III
B. anthracis BA1018	LANL, NM, USA	III
B. anthracis CDC-572	LANL, NM, USA	III
B. anthracis SG-PA3	LANL, NM, USA	III
B. anthracis Ames	LANL, NM, USA	III
Bacillus thuringiensis 127	LANL, NM, USA	III
B. thuringiensis 314	LANL, NM, USA	III
B. thuringiensis 487	LANL, NM, USA	III
B. thuringiensis 780	LANL, NM, USA	III
B. thuringiensis 10792	LANL, NM, USA	III
Yersinia enterocolitica	LANL, NM, USA	III
Yersinia pestis Angola	LANL, NM, USA	III
Y. pestis Antigua	LANL, NM, USA	III
Y. pestis CO92	LANL, NM, USA	III
Y. pestis Ev76-lot4	LANL, NM, USA	III
Y. pestis Ev76-51	LANL, NM, USA	III
Y. pestis Harbin 35	LANL, NM, USA	III
Y. pestis Indian Isolate	LANL, NM, USA	III
Y. pestis Java 9	LANL, NM, USA	III
Y. pestis Kim-10-Variant	LANL, NM, USA	III
Y. pestis KIM10	LANL, NM, USA	III
Y. pestis La Paz	LANL, NM, USA	III
Y. pestis Nicholisk 41	LANL, NM, USA	III
Y. pestis Pest A	LANL, NM, USA	III
Y. pestis Pest Aa	LANL, NM, USA	III
Y. pestis Pest B	LANL, NM, USA	III
Y. pestis Pest Ba	LANL, NM, USA	III
Y. pestis Pest C	LANL, NM, USA	III
Y. pestis Pest D	LANL, NM, USA	III
Y. pestis Pest F	LANL, NM, USA	III
Y. pestis Pest G	LANL, NM, USA	III
Y. pestis Pest J	LANL, NM, USA	III
Y. pestis Russian Plague Vaccine	LANL, NM, USA	III
Y. pestis Stavropol	LANL, NM, USA	III
Y. pestis III	LANL, NM, USA	III
Y. pestis 195P	LANL, NM, USA	III
Yersinia pseudotuberculosis	LANL, NM, USA	III

LANL = Los Alamos National Laboratory

Table 4. Plasmids used in this study.

Plasmid	Relevant properties	Reference/source	Used in
pGFPuv	Amp^r, gfp_{uv}, *Nco*I, *Eco*RI, *Ava*II	Clonetech Laboratories Inc./ Anonymous 2000	IV
pCR®4-TOPO	Kam^r, *Eco*RI, *Not*I	Invitrogen Life Tecnologies	IV
pNZ8048	Cam^r, P_{nisA}, *Nco*I, *Eco*RI, terminator	NIZO/Kuipers et al. 1998	IV
pLEB651	Cam^r, $P_{nisA,}$ gfp_{uv}, *Eco*RI	This study	IV

Amp = ampicillin, Kam = kanamycin, Cam = chloramphenicol, r = resistance

Table 5. Restriction endonucleases used in this study.

Enzyme	Base sequence	Used in
*Ava*II[a]	5'- G*G(A/T)CC -3' 3'- CC(T/A)G*G -5'	IV
*Bsp*HI	5'- T*CATGA -3' 3'- AGTAC*T -5'	IV
*Eco*RI	5'- G*AATTC -3' 3'- CTTAA*G -5'	IV, V
*Hin*dIII	5'- A*AGCTT -3' 3'- TTCGA*A -5'	III
*Nco*I	5'- C*CATGG -3' 3'- GGTAC*C- 5'	IV
*Not*I	5'- GC*GGCCGC -3' 3'- CGCCGG*CG -5'	I, II
*Sfi*I[b]	5'- GGCCNNNN*NGGCC- 3' 3'- CCGGN*NNNNCCGG- 5'	I
*Xba*I	5'- T*CTAGA- 3' 3'- AGATC*T- 5'	I
*Sma*I	5´- CCC*GGG -3' 3'-GGG*CCC- 5'	II

[a]parentheses indicates that can be either base
[b]N indicates any base

Table 6. Sequences of PCR primers used in this study.

Primer name and restriction site	Sequence 5'→ 3'	Used in
*Hin*dIII + 0	GTAGACTGCTGACCAGCTT	III
*Hin*dIII + XX	GTAGACTGCTGACCAGCTTXX[a]	III
G0575	AGCTGCATGTGTCAGAGGTTTTCA	IV
G0576, *Bsp*HI	AGAAATCATGAGTAAAGGAGAAGAAC	IV
pA (forward primer)	AGAGTTTGATCCTGGCTCAG	V
pE' (reverse primer)	CCGTCAATTCCTTTGAGTTT′	V

[a]XX represents one or two additional specific nucleotides: A, C, G, T, AC, AG, AA, AT, CA, CT, GA, GT, TA, TC, TG, and TT

Table 7. Methods used in this study.

Method	Used in
Basic DNA techniques: DNA isolation, plasmid isolation, restriction enzyme digestion, electrophoresis, PCR	I-V
DNA isolation by agarose plug method	I, II
Staining: DNA, spermatozoa	I, II, V
DNA electroelution	I, II
DNA fingerprint fragment analysis	I, II, III
Transformation: *E. coli* and *L. lactis*	IV
Nisin quantification by nisin-induced GFP_{uv} fluorescence	IV
Partial 16S rRNA gene sequencing	V
Pulsed-field gel electrophoresis	I, II
Riboprinting analysis	V
SE-AFLP	III
Boar spermatozoa toxicity assay	V
Ultrasensitive flow cytometry	I, II

RESULTS AND DISCUSSION

1. BACTERIAL FINGERPRINTING WITH FLOW CYTOMETRY (I, II)

PFGE has been accepted as the primary molecular typing method in the United States, PulseNet, and Europe, PulseNet Europe, for recognition and investigation of foodborne outbreaks (Gerner-Smidt et al. 2006; Rodríguez-Lázaro et al. 2007). However, PFGE requires relatively large quantities of DNA, the sample preparation is time consuming and the analysis can be difficult. Therefore, a method that requires less DNA and is quicker is an advantage for bacterial discrimination and identification.

1.1. Technique of DNA fragment sizing with FCM

In this study, a bacterial identification method was developed based on the analysis of the DNA fragments by an ultrasensitive flow cytometer. The DNA fragments were obtained by digestion of chromosomal DNA in an agarose plug with a restriction enzyme as with PFGE. However, rather than characterizing the DNA fragment sizes by gel electrophoresis, the DNA fragments were electroeluted from the plug and stained with a DNA intercalating fluorescent dye. The stained fragments were then analyzed by an ultrasensitive flow cytometer that is capable of single molecule detection (Goodwin et al. 1993; Petty et al. 1995; Huang et al. 1996; Huang et al. 1999). Each fragment produced a fluorescence burst, directly proportional to the length, due to the excitation by a laser. These bursts were detected, recorded, and a histogram was produced from these burst sizes, which was equivalent to the DNA fingerprint received by PFGE. High efficiency optics and light detection, bright fluorochromes, and slow sheath velocity were used to develop the ultrasensitive flow cytometry system (Goodwin et al. 1993; Petty et al. 1995; Huang et al. 1996; Huang et al. 1999). By having a slow sheath velocity of 1-4 cm/s compared to the usual velocity of 1000 cm/s, allowed the DNA fragments to spend longer time in the laser beam, and therefore for better detection of the emitted fluorescence of the sample. The instrument contained a continuous wave Ar^{+}/Kr^{+} laser to excite the dyed DNA fragments. DNA fragment analysis by flow cytometric measurements has also been reported by others (Castro et al. 1993; Goodwin et al. 1993; Castro and Shera 1995; Petty et al. 1995; Huang et al. 1996; Schins et al. 1998; Agronskaia et al. 1999; Chou et al. 1999; Huang et al. 1999;), but in this study (Paper I and II) multiple bacterial species, *Bacillus globigii, E. coli, Erwinia herbicola* and *S. aureus,* and also five different strains of *E. coli* and six different strains of *S. aureus*, were investigated with the FCM as a DNA fragment sizing method.

The DNA fragments were stained with fluorescent intercalating dyes, TOTO-1 (Paper I) and PicoGreen (Paper II), which bind stoichiometrically to DNA. Thus, the fragment size was directly proportional to the bound dye. The intercalated dye fluoresces up to thousand times higher making it unnecessary to wash the unbound dye (Glazer and Rye 1992; Yan et al. 1999), and in addition increasing the sensitivity of the method. In FCM analysis, only 1-2 picograms of DNA were required compared to the few hundred nanograms usually used for PFGE. Further developments have been made in the DNA staining techniques for the DNA fragment sizing by flow cytometry. For example, Yan et

al. (2000) developed a universal DNA staining protocol using SYTOC Orange stain that had high fluorescence when bound to DNA and was independent of the staining dye to DNA base pair ratio, which caused problems in previous staining techniques used with FCM.

In order to obtain the best resolution between DNA fragments, the endonucleases must not cut the DNA at too many sites which would result in a large number of total fragments that cannot be clearly separated. As in the case with *B. globigii* digested with *Sfi*I, the flow cytometer could not resolve the individual fragments (Paper I) and therefore fragment size determination was not possible. Therefore, the choice of the proper endonuclease is crucial in the fingerprinting analysis by flow cytometry.

1.2. Accuracy, reproducibility and sensitivity of FCM

The ultrasensitive flow cytometer described in this study measured DNA fragments ranging from 20-425 kbp. Larger fragments were present in the restriction enzyme digestion, as evident by the PFGE, but FCM was not able to detect these fragments due to breakage of DNA. Even though all DNA fragments could not be analyzed by the FCM, unique histograms that were comparable to PFGE results were obtained for different species and strains. According to Huang et al. (1996) and Goodwin et al. (1993), the uncertainty for FCM in size determination is approximately 2% while for PFGE it is 10%. In this study, the uncertainty in the DNA size determination was within the 10% when comparing the results between FCM and PFGE. Thus, DNA fragment sizing by FCM is reliable and comparable to PFGE.

During a seven month period, histograms of different preparations of *S. aureus* ATCC 25923 digested with *Sma*I were collected to study the reproducibility of the flow cytometric measurements (Paper II). The fingerprints obtained from different cultures, different sample preparations from the same culture, and different sample analyses were identical. In addition, DNA stored in agarose plugs for several months or in solution for one month had reproducible results.

The FCM analyses of DNA fragment sizes were based on calibration with PFGE results, but in a case of an unknown bacterium, PFGE data is not available. Therefore, an internal standard was developed for the FCM to allow calibration of the burst sizes with fragment length (Paper I). Lambda-DNA was added to the analyte as an internal standard to obtain a calibration curve of 0 kbp (no λ), 48.5 kbp (λ) and 97.0 kbp (two λs), from which two points were used to convert the centroid positions of the burst peaks of FCM to kilobase pairs. With the internal standard, the fragment sizes were still within the 10% comparison to PFGE results and can be used in the sizing of DNA fragments of unknown bacterium.

The accuracy and precision of the FCM method as compared to PFGE were further assessed by Ferris et al. (2004). They compared the DNA fragment sizes of replicate samples of *S. aureus* Mu50, (completely sequenced genome) and two clinical *S. aureus* isolates digested with *Sma*I by the two methods. According to their results, the accuracy

(percent error from the virtual digestion to the fragment sizes calculated by the specific DNA sizing method) was 5% ± 2% for PFGE and 4% ± 4% for FCM measurements. The precision (consistency in the sizing of the restriction fragments in repeated analyses) was calculated as mean relative standard deviation values, which were found to be 1.2% ± 0.8% and 3% ± 2% for FCM and PFGE, respectively. These values are in agreement with the results previously reported in literature (Goodwin et al. 1993; Huang et al. 1999; Duck et al. 2003), including Papers I and II.

1.3. Rapid fingerprinting of bacteria by FCM

The actual analysis time of the DNA size determination by FCM takes about 10 minutes compared to the 15-20 hours by PFGE. However, the DNA sample preparation by the agarose plug method and the overnight electroelution of the digested DNA from the plug results in the overall analysis time similar to that of PFGE. Therefore, a quicker and simpler protocol (Paper II) was developed for the FCM method. By using more effective lysozyme, reducing the time of the steps of the procedure by decreasing the size of the agarose plug, and eliminating the electroelution step by digesting the agarose by GELase, the procedure was shortened to eight hours compared to the traditional DNA isolation procedure by agarose plug taking three to eight days. Although others have described four and eight hours DNA isolation by agarose plug procedures for PFGE (Gautom 1997; Chang and Chui 1998), these DNA preparation still require the electroelution of DNA for FCM analysis. Therefore, with the short protocol, fragment analysis can be done in approximately 8 hours by flow cytometry rather than 24 hours or longer by PFGE.

In conclusion, the DNA fragment sizing by FCM is accurate, reliable, and rapid when compared to the golden standard for bacterial fingerprinting, PFGE. Although with FCM only one sample can be analyzed at a time and with PFGE multiple sample lanes can be run at in parallel, the analysis time is hundred times faster with FCM. In addition, the FCM method requires approximately 200,000 times less DNA compared with PFGE. Even though DNA fragment sizing analysis by the ultrasensitive flow cytometry is an excellent alternative for PFGE, at present, it is not available commercially. However, it is available to the scientific community through the National Flow Cytometry Resource at Los Alamos National Laboratory.

2. SINGLE-ENZYME AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (III)

The conventional method of amplified fragment length polymorphism (AFLP) uses two restriction enzymes (Vos et al. 1995) resulting in fingerprints with hundred or more fragments of 100-500 bp long. The pattern identification is difficult and therefore special software is necessary to analyze the fingerprints and to compare with an AFLP fingerprint library. In this study, a single-enzyme amplified fragment length polymorphism (SE-AFLP) was used to minimize the number of fragments and therefore simplify the comparison of fingerprints (Fig. 5). SE-AFLP has also previously been used for differentiation of *B. cereus* (Ripabelli et al. 2000b), *Chlamydia psittaci* (Boumedine and

Rodolakis 1998), *Helicobacter pylori* (Gibson et al. 1998), *Legionella pneumophilia* (Valsangiacomo et al. 1995), *L. monocytogenes* (Ripabelli et al. 2000a), and *S. enterica* (Peters and Threlfall 2001). In this study, the SE-AFLP method was further investigated as an identification method for different strains of *Bacillus* spp., *E. coli*, *Staphylococcus* spp., and *Yersinia* spp..

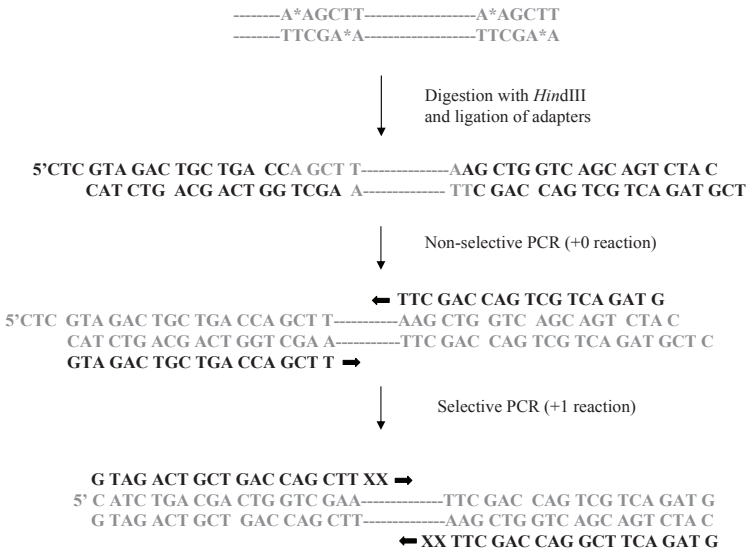

Fig. 5. Single-enzyme amplified fragment length polymorphism with *Hin*dIII as performed in Paper III. The restriction site is indicated by * and adapters and primers in black.

The primers *Hin*dIII + AC and *Hin*dIII + G were found to be the best primers for the selective PCR amplification step since it was able to distinguish between the very monomorphic *B. anthracis* strains Ames and SG-PA3. Both of these primers, if *Hin*dIII + AC primer was not alone sufficient, were tested for their capability of differentiating between species and strains of *Bacilli, Escherichia, Staphylococci,* and *Yersinia*. For *E. coli* and *S. aureus,* primer *Hin*dIII + AC was capable of discriminating between strains and subspecies. With *E. coli,* the pathogenic strains of serotype O157 ATCC 43888 and 43895 were differentiated from the other strains, but their fragment patterns were identical to each other. This was also the case with the non-pathogenic *E. coli* strains, MG 1655, ATCC 25254, and ATCC 27662, which is not surprising since they are derived from *E. coli* K-12. The *S. aureus* subspecies *aureus* strains ATCC 29996 and ATCC 25923 patterns were different from each other and from the subspecies *anaerobius,* ATCC 13301, ATCC 10832, and ATCC 12600. Unfortunately, the patterns within the *S. aureus* subspecies *anaerobius* were identical to each other. However, the interesting result was that the fingerprinting pattern of *S. aureus* subspecies *aureus* ATCC 29213 was more closely similar to the subspecies *anaerobius* and not to its subspecies *aureus*. With *Yersinia* spp., SE-AFLP using *Hin*dIII + AC and *Hin*dIII + G primers, were able to differentiate between *Yersinia enterocolitica*, *Y. pestis* and *Y. pseudotuberculosis*. Within *Y. pestis*, the strains were not differentiated even with multiple combinations of *Hin*dIII + X primers, but they could be categorized into ten different groups based on

the SE-AFLP fingerprint patterns with the combination of *Hin*dIII + AC and *Hin*dIII + G primers. From the 25 total strains of *Y. pestis,* fifteen strains grouped into two specific groups suggesting that *Y. pestis* is monomorphic. The closely related species within the *Bacillus* subgroup I (*B. anthracis, B. cereus, B. thuringiensis* and *B. mycoides*) were also studied by using SE-AFLP. The primer *Hin*dIII + AC was able to differentiate strains within *B. cereus, B. thuringiensis* and *B. mycoides*. However, the strains of *B. anthracis* were difficult since there was only slight, if not at all, differences in the fingerprinting patterns. Among 78 *B. anthracis* strains, 97% fragment identity has been observed when studied by conventional AFLP method (Keim et al. 1997).

Even though SE-AFLP appeared to produce various DNA fingerprint patterns, it is not capable of identifying all strains within a species such as *B. anthracis*. However, it improved the differentiation of species and strains within polymorphic bacterial groups. Furthermore, it is highly reproducible as shown by analyses of three separate preparations of *B. thuringiensis* ATCC 10792 (standard deviation ranged from 0.007 - 0.229 for fragment sizes).

3. GFP_{UV}-BASED NISIN BIOASSAY (IV)

Nisin is a ribosomally produced antimicrobial peptide, a bacteriocin, produced by lactic acid bacteria (O'Sullivan et al. 2002). Since it inhibits the growth of other gram-positive bacteria, including food-borne pathogens, such as *B. cereus, C. botulinum, C. perfringens, L. monocytogenes* and *S. aureus*, and it is regarded as GRAS (generally recognized as safe; Thomas et al. 2000), over fifty countries worldwide allow it as a food preservative (E234; Delves-Broughton et al. 1996; EEC 1983). It is suitable for many different types of food, but commonly used in dairy products, such as processed cheese, cheese spreads, and puddings, but it is also used in salad dressings, vegetables and even beer (Anonymous 2002; Delves-Broughton et al. 1996). However, the regulations on the amount and foods that nisin can be added to vary by region (Anonymous 2002). Therefore, it is necessary to have methods that quantify nisin from different food products.

In this study, a new indicator *Lactococcus lactis* strain*,* LAC275, was constructed to create a sensitive nisin quantification detection system. The indicator strain carries the plasmid, pLEB651, with the gfp_{uv} gene encoding an ultraviolet variant of the green fluorescent protein under the control of the nisin-inducible *nisA* promoter (Fig. 6). The plasmid pLEB651 was introduced to *L. lactis* NZ9000 that contained in its chromosome the nisin signal transduction system *nisRK* genes.

The LAC275 cells sense nisin in the environment and respond by activating the two-component signal transduction proteins NisK and NisR, which enduces the expression of the GFP_{uv} (Fig. 7). The fluorescence can be measured by a fluorometer and be correlated with the amount of nisin in the environment. The constructed *L. lactis* LAC275 strain was able to detect extracellular nisin from various different food matrices: culture medium, milk, processed cheese, salad dressings, canned tomatoes and liquid egg.

Other methods, based on biochemical reactions, immunologic detection, growth inhibition in liquid and solid media, and bioluminescence have been used to quantify nisin, but most of these methods are not as sensitive as LAC275 and have not been tested with multiple different food products (Table 8). Time of flight mass spectrometry (Hindré et al. 2003), flow cytometry (Budde and Rasch 2001), and capillary zone electrophoresis (Rossano et al. 1998) have also been used to detect and quantify nisin. Many of these other detection methods are difficult to perform, not specific for nisin, and/or their sensitivity is not sufficient. Biochemical reactions and agar diffusion methods are based on the inhibitory actions of nisin, but they are not exclusively for detecting nisin (de Vuyst and Vandamme 1994). Immunological methods have similar specificity problems, such as cross-reactivity with lantibiotic subtilin (Falahee and Adams 1992) or other variants of nisin, such as nisin Z (Suárez et al. 1996b). In other

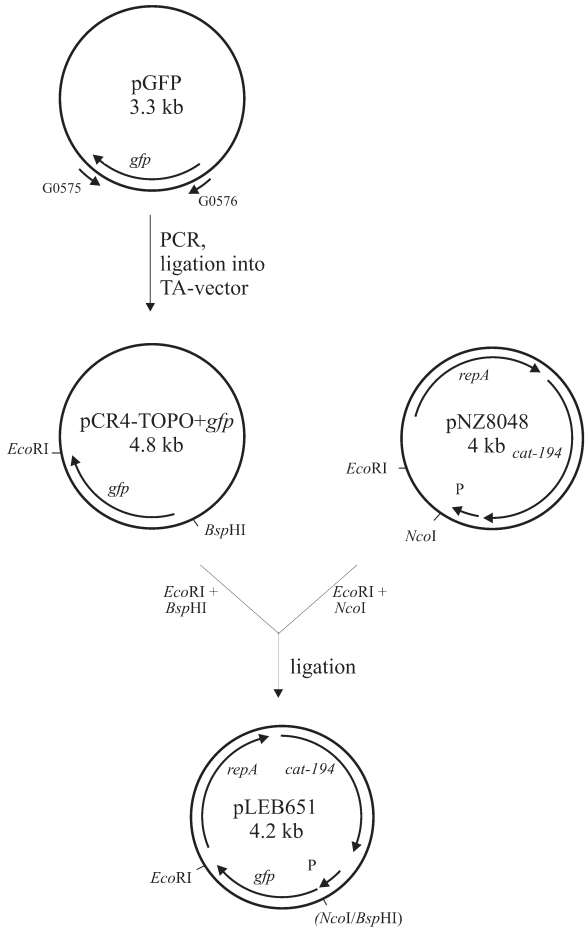

Fig. 6. Schematic representation of the construction of the plasmid pLEB651. The figure was compiled from de Ruyter et al. 1996, Kuipers et al. 1998, and Anonymous 2000. P indicates the promoter *nis*A and *gfp* indicates the gene encoding the ultraviolet variant of the green fluorescent protein.

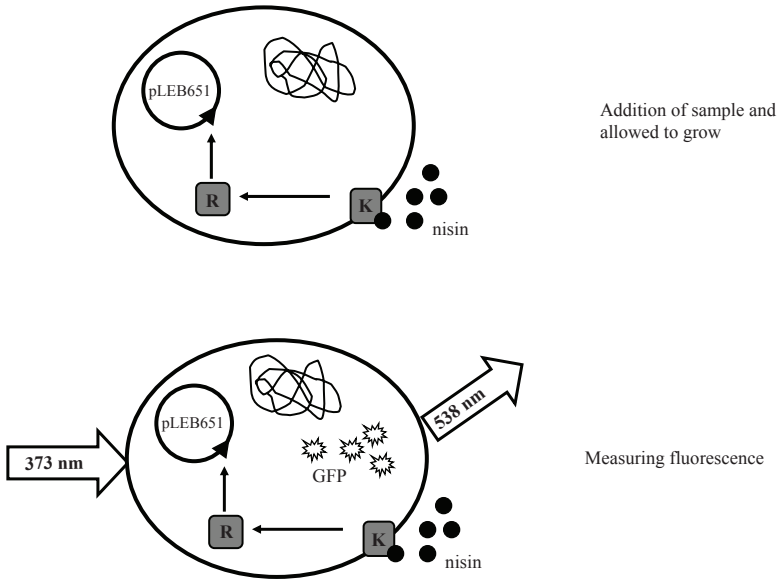

Fig. 7. The GFP_{uv} fluorescence bioassay for nisin with the indicator strain *L. lactis* LAC275. Sample is inoculated with LAC275 and incubated overnight at 30ºC on a microplate. The supernatant is removed from the top of the cell pellet and the fluorescence is measured with excitation at 373 nm and emission at 538 nm.

cases, nisin quantification may be difficult to perform for simultaneous analysis of multiple samples due to specific requirements of the method. For example, a luciferase assay using bioluminescence genes of *Xenorhabdus luminescens, luxAB,* under the control of the *nisF* promoter and regulated by the NisR and NisK proteins required the addition of a substrate to the cells at a specific growth stage (Wahlström and Saris 1999). Immonen and Karp (2007) improved the luciferase assay by using the complete set of luciferase genes from *Photorhabdus luminescens*, *luxABCDE*, under the control of the *nisA* promoter. This simplified the assay so that the luciferase substrate was not required to be added to the cells at a specific growth stage. Not only was the procedure simplified, but also the overall time needed to perform the assay was shortened to three hours and the detection limit for nisin quantification was reduced to 0.1 pg/ml in pure solution and 3 pg/ml in milk making it the most sensitive nisin detection system presently available. The sensitivity of the nisin bioassay applying GFP_{uv} allows extensive dilution of foods, thus minimizing materials which could interfere with the analysis. The method also allows the processing of multiple samples at the same time and has been shown to be compatible with different food matrices.

Table 8. The various nisin detection and quantification methods and their detection limits in various matrices.

Method	Detection limits for nisin (ng/ml)							
	Pure solution*	Canned tomatoes**	Cheese**	Liquid egg**	Milk	Salad dressing**	Sausage**	Whey
ELISA for NisA[a]	0.5		1250					
CD-ELISA[1] for NisZ[b] (monoclonal antibodies)	10		250					
CD-ELISA[1] for NisZ[c] (polyclonal antibodies)	5							
Dot-blot immunoassay for NisZ[d]	375				155			155
ELISA for NisZ[e]	0.75/ 3.5[2]				1.7			
Competative enzyme nisin immunoassay for NisZ[f]	78/ 87[2]				106			90.5
Agar diffusion[g]	12.5							
luxAB bioluminescence assay[h]	0.0125[3]				1			
GFP-based bioassay[i]	2.5[4]		900		45	1000	900	
GFP_{uv}-based bioassay[j]	0.01[4]	1	3.6	9	0.9	1		
luxABCDE bioluminescence assay[k]	0.0001				0.003			

[1]CD-ELISA, competitive direct enzyme-linked immunoabsorbent assay

*detection limit in pure solution unless otherwise indicated; [2] de Man, Rogosa, and Sharpe broth; [3] in pure solution and M17 broth containing 0.5% (wt/vol) glucose and sucrose; [4] M17 broth containing 0.5% (wt/vol) glucose

**in ng/g

[a] Falahee et al. 1990; [b] Suárez et al. 1996b; [c] Suárez et al. 1996a; [d] Bouksaim et al. 1998; [e] Bouksaim et al. 1999; [f] Daoudi et al. 2001; [g] Tramer and Fowler 1964; [h] Wahlström and Saris 1999; [i] Reunanen and Saris 2003; [j] This study (Paper IV); and [k] Immonen and Karp 2007.

4. MICROBIOTA OF CUSTOMER RETURNED WINES (V)

Wine is considered to be bacterially safe from the common food-related pathogens. The antimicrobial characteristic of wine has been shown by others by inoculating certain bacteria, *E. coli, L. monocytogenes, Shigella* spp*., Salmonella* spp., *S. aureus* and *Vibrio parahaemolyticus* into wines; they did not survive for long time in a such harsh environment (Weisse et al. 1995; Harding and Maidment 1996; Marimón et al. 1998; Mugochi et al. 1999; Sugita-Konishi et al. 2001; Just and Daeschel 2003; Møretro and Daeschel 2004). Although these studies have indicated that these bacteria could not expose a risk to wine consumers, customers have returned wines due to food poisoning symptoms. In this study, twelve customer returned wines were analyzed for their microbial content by partial 16S rRNA gene sequence analysis to find out a possible cause for these customer complaints.

Microbial content analysis of wines was difficult since isolation of bacteria from this matrix was problematic. In many cases more colonies were isolated for each wine sample, but not all of them could be further analyzed since they grew poorly or not at all immediately or soon after the preparation of the pure culture. These types of difficulties have also been reported by others who have studied microbial content. Millet and Lonvaud-Funel (2000) reported that wine contains VNC microorganisms and those bacteria that survive the wine environment appear to grow much slower and form smaller colonies than bacteria that have not been exposed to this stressful condition. In order to study these minority species, more efficient growth media have been developed (Renouf and Lonvaud-Funel 2006), but this in return can eliminate other microbes that are present in the wine. We randomly picked and isolated 37 bacteria from the wine samples. The partial 16S rRNA gene was amplified by using universal primers pA and pE' (Edwards et al. 1989) for 16S rRNA gene sequence determination and analysis. All partial 16S rRNA gene sequences were classified by using the Ribosomal Database Project II (RDP) Classifier (version 2.0) to the 80% confidence level for genus identification with the new phylogenetically consistent higher-order bacterial taxonomy proposed by Wang et al. (2007a). The twelve wine samples contained bacteria belonging to *Bacillus* spp., *Pedioccocus* spp., *Paenibacillus* spp., *Acetobacter* spp., *Enhydrobacter* spp., *Gluconobacter* spp., *Micrococcus* spp., *Kocuria* spp., *Dermabacter* spp. and *Rothya* spp.. The interesting finding was that almost half of the sampled wines contained *Bacillus* spp. and 15 of the 37 bacterial isolates were identified as *Bacillus* spp. It is not surprising to find *Bacillus* spp. in wine since they are common in the environment and their ability to form spores allows them to survive stressful environments (Henriques and Moran 2007). In addition, Bae et al. (2004) have shown that if *B. thuringiensis*, present in insecticides, is sprayed on wine grapes, they survive the wine making process and end up in the final product. However, the amount of samples containing bacilli raised our curiosity to further study these isolates, especially since the genus *Bacillus* includes species that can produce toxins and can cause food-poisoning, such as *B. cereus* (Granum 2007).

The basic local alignment search tool BLAST was used to compare the partial 16S rRNA gene sequences of the fifteen *Bacillus* spp. isolates from wine against the nucleotide database in National Center for Biotechnology Information. Seven of the fifteen *Bacillus* spp. isolates were identified as *Bacillus flexus* (n=5), *B. simplex* (n=1) and *B. megaterium* (n=1) with sequence similarity of ≥ 98.7% proposed by Stackebrandt and Ebers (2006) for species identification. *Bacillus* sp. BAC91 isolated from wine had 16S rRNA gene sequence similarity of 100% to the type strains *B. simplex* DSM 1321 and *Bacillus muralis* DSM 16288. Therefore according to 16S rRNA gene sequence analysis, *Bacillus* sp. BAC91 can belong to either species. The seven other *Bacillus* spp. isolates had sequence similarities < 98.7% and thus could not be identified at the species level. Others have also reported difficulties in identification of bacterial isolates by 16S rRNA gene sequence analysis. Drancourt et al. (2000) described a broad study for 177 isolates obtained from environmental, veterinary, and clinical sources where 90% of the phenotypically unidentified bacteria were identified by 16S rRNA gene sequence analysis at least to the genus level. However, isolates belonging to *Bacillus* spp. and *Enhydrobacter* spp. were more difficult to identify to the species level. Species identification failure by 16S rRNA gene sequencing has also been reported for enteric bacteria (Mollet et al. 1997). The inability of identifying *Bacillus* spp. to the species level is not unusual since they can have very similar or in certain cases almost identical 16S rRNA gene sequences (Ash et al. 1991; Fox et al. 1992).

For further characterization of *Bacillus* sp. BAC91, riboprinting was performed as described by Pirttijärvi et al. (1999) with *Eco*RI using an automated RiboPrinter™ Microbial Characterization System and the Riboprinter™ 2000 System Data Analysis Program. The ribopattern of *Bacillus* sp. BAC91 was compared to the ribopatterns of various different *Bacillus* spp. type strains and their representative toxin producing strains by analyzing the patterns with UPGMA (unweighted pair-group mean arithmetic means) using Pearson's correlation similarity coefficients. The *Bacillus* sp. BAC91 clustered with *B. simplex* with 96% similarity to the type strain *B. simplex* DSM 1321 in the UPGMA dendrogam, which is above the 88% ribopattern similarity for species identification (Suihko and Stackebrandt 2003). With high similarity to the type strain´s *B. simplex* DSM 1321 16S rRNA gene sequence and ribopattern, the *Bacillus* sp. BAC91 belongs to *B. simplex.*

Since the genus *Bacillus* includes species that can produce toxins and can possibly cause food-illnesses, all fifteen *Bacillus* spp. isolates were tested for their production of toxic substances by the boar spermatozoa motility assay (Andersson et al. 1998; Andersson et al. 2004). *B. simplex* BAC91 was shown to produce a heat-stable, toxic substance that affected the motility of the sperm cells. The exposed sperm cells were further inspected for the effect of the toxic substance on the mitochondrial inner membrane potential and cell membrane integrity by fluorescent dyes (Hoornstra et al. 2003) JC-1 (mitochondrial stain), propidium iodide and calcein acetoxy methyl (plasma membrane integrity). The toxic substance was found to cause similar damage to the mitochondria of the spermatozoa as cereulide and valinomycin described by Hoornstra et al. (2003). Not much is known about toxin-producing *B. simplex* and their properties, especially

in foods. However, Taylor et al. (2005) and Peltola et al. (2001) have reported of toxin-producing *B. simplex* in a cystic fibrosis patient and from the indoor environment of a moisture damaged building, respectively.

The bacteriocidal and sporacidal potential of wine and grape juice was also assessed for *B. simplex* BAC91, *B. cereus* F4810/72 (cereulide-producer), and *B. cereus* type strain ATCC 14579. The viability of the three bacteria varied when they were exposed to red wine, white wine, and grape juice. Overall the vegetative cells of *B. cereus* F4810/72 and spores of *B. cereus* F4810/72 and *B. cereus* ATCC 14579 were the most resistant to the antimicrobial effects of wine. The vegetative cells and spores of *B. simplex* BAC91 were the most sensitive even though the strain had been isolated from wine. It is possible that after isolation from the wine and growing it in laboratory conditions, the re-introduction of the *B. simplex* BAC91 into the wine environment caused them to become VNC, which has been described for other wine microorganisms (Millet and Lonvaud-Funel 2000).

Wines can become easily contaminated by bacilli through the raw materials or during the making process. Bacilli are common in the environment (dust, soil) and even used in insecticides, such as *B. thuringiensis* (Bae et al. 2004), and therefore can contaminate the grapes used in wine fermentation. In addition, during the wine making process, bacilli can be introduced to it from the pipes and storage containers, which have been shown to be excellent surfaces for formation of spore-rich biofilms (Wijman et al. 2007). Since *Bacillus* spp. were found in half of the customer returned wine samples and that spores of *B. cereus* can survive in grape juice and wine, this further suggest that if bacilli, more specifically spores, contaminate wine, they can be a possible risk to the consumer. The risk is not necessarily in the direct consumption of wine, but perhaps more when wine is used in cooking. The heating of wine can cause the spores to germinate, grow, and possibly produce toxins in the food, which can result in food poisoning as in the case with *B. cereus*. However, to fully assess the risk of bacilli in wine, a broader study with larger amounts of wine samples is needed. Also, including culture-independent methods in the microbial content analysis would give a better understanding of the actual microbial population in wine, which are not detectable by culture-dependent methods.

CONCLUSIONS

In emergency cases, such as food epidemics or biological warfare, detecting and identifying quickly the unknown organism(s) that is causing the illness is crucial. These detection and identification methods must be simple and quick, yet sensitive and reproducible. PFGE is considered to be the "golden method" in identifying the causative organism in cases of food, water and hospital epidemics. Although the method is reliable and accurate, the sample preparation and analysis are time consuming. FCM was studied as an alternative for PFGE for bacterial subtyping analysis. In this thesis, FCM bacterial fingerprinting discriminated species and strains in a reproducible and comparable manner to PFGE. The FCM method was hundreds times faster and 200,000 times more sensitive. Another DNA fingerprinting identification method is AFLP, but the conventional double enzyme AFLP produces results that are difficult to analyze without appropriate software and databases. By SE-AFLP, the resulting fragment patterns were simplified and the genera, species and strains of pathogenic bacteria of *Bacilli, Staphylococci, Yersinia* and *E. coli* could be differentiated. However, monomorphic species, such as *B. anthracis* and *Y. pestis*, could only be identified at the species level.

Food preservatives and food additives are components of food that need regulation and control. Nisin (E234), for example, is a food preservative that is allowed to be added to different types of food, especially dairy products, around the world. Various detection methods exist for nisin, but they lack either sensitivity, speed or specificity. In this thesis, a sensitive nisin-induced GFP_{uv} bioassay was developed using the *L. lactis* two-component signal system NisRK and the nisin-inducible *nisA* promoter. The bioassay was shown to be compatible with analyzing nisin from various food matrices and was shown to be extremely sensitive with detection limits ranging from 10 pg/ml in culture medium to 3.6 ng/g in cheese.

A common belief is that wine is free of bacteria that can cause food poisoning. However a heat-stable mitochondriotoxin producing *B. simplex* BAC91 was isolated from a customer returned wine. Although vegetative cells and spores of *B. simplex* BAC91 were sensitive to the antimicrobial effects of wine, spores of *B. cereus* strains ATCC 14579 and F4810/72 remained viable. These results are worrisome since this suggests that if wine becomes contaminated with *Bacillus* spp., particularly spores, they can survive to the final product and be a potential risk to consumers.

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