IDENTIFICATION OF MICROORGANISMS IN FOOD ECOSYSTEMS AND
CHARACTERIZATION OF PHYSICAL AND MOLECULAR EVENTS
INVOLVED IN BIOFILM DEVELOPMENT

Dissertation

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ABSTRACT

Most foods can be considered as ecosystems containing various microorganisms including pathogenic, spoilage, commensal microbes and fermentation starter cultures. Microbial biofilm ecosystems also form on the surfaces of processing equipment. The interactions among microbes and between microbes and various surfaces play an important role in the persistence and the prevalence of these microbes in the food environment. Diversity of food matrices adds complexity to and directly shapes the composition of the microbiota in these ecosystems. Proper identification and quantification of microbes, evaluation of potential risks in the food ecosystems, and characterization of the physical and molecular events involved in ecosystems, including biofilm development, are among the primary tasks for food microbiologists. The objectives of this study are to develop a rapid detection system for foodborne microorganisms using molecular approaches, to characterize component(s) involved in biofilm development, and to examine the contribution of commensal organisms in ecosystem development and horizontal gene transfer.

A real-time PCR system was developed to rapidly detect Alicyclobacillus spp. and Listeria monocytogenes in food. Detection of less than 10 bacterial cells per reaction was achieved within 4-7 hours. CluA, a surface protein related to cell clumping, was found to
be an important component in the development of *Lactococcus lactis* biofilm. Biofilm attributes can be disseminated within the ecosystem by conjugation, and a lactococcal strain carrying the intrinsic high frequency gene transfer mechanism could increase the transfer of pAMβ1 by 10,000-fold. The study demonstrated, for the first time, that conjugation facilitated biofilm formation in Gram-positive bacteria, and that commensal organisms not only served as a gene pool but also as enhancers facilitating horizontal gene transfer, including the dissemination of the drug resistance genes. Finally, our data showed that commensal organisms, such as *Pseudomonas*, enhanced mixed-culture biofilm formation involving other microorganisms such as *Staphylococcus*, suggesting the contribution of commensal organisms in pathogen persistence.

The rapid detection system developed in this study, has direct applications in both food industry and basic scientific research. Results from this study advanced the knowledge in biofilm development and gene transfer mechanisms.
Dedicated to Qingyue Guo, my husband,
for his love and support.
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CHAPTER 1

INTRODUCTION

Foodborne microorganisms play an important role in our daily life. Starter cultures, for example, are used in food fermentation. They add desirable features to the products, including improved flavor, texture and nutritional values. Fermentation is also an effective way to preserve perishable food products. Probiotics are considered beneficial microorganisms due to their potential role in improving the overall health of animal or human host. The contribution of commensal organisms in ecosystems is yet to be revealed. However, the presence and outgrowth of foodborne pathogens and spoilage organisms in foods significantly impact product safety and quality, causing losses to the food industry and the community. According to World Health Organization (WHO), there are 76 million cases of foodborne illness in the US, resulting in 325,000 hospitalizations and 5000 deaths annually (www.who.int/foodsafety/micro/general/en/). Over the past several decades, significant increases in foodborne illnesses caused by microorganisms have also been documented in other countries. Meanwhile, it is estimated that up to 25% of all food produced in the United States are lost annually, mainly due to growth of spoilage microorganisms (Ray 2001; www.usda.gov/news/releases/1997/07/0212). Improving the safety and quality of
food products through controlling the microbial activities in the food environment is therefore a primary task for food microbiologist.

While sterilization could eliminate viable microorganisms in the final products, extreme processing conditions often cause undesirable sensory changes and loss of nutritional values. Therefore assuring food safety and quality still largely relies on proper monitoring the microbial quality in raw materials, food processing environment, and final products.

Conventional industrial practices for microbial detection cannot fulfill the requirements of the food industry, especially for foods with limited shelf life. For example, the thermophilic and aciduric Gram-positive, spore-forming *Alicyclobacillus* is becoming an important spoilage concern in juice products. However, the current detection methods including conventional culturing and biochemical analysis, take at least 2 days for results delivery, while the shelf life of juice products is only about two weeks. *Listeria monocytogenes* can cause listeriosis in susceptible individuals, with a mortality rate of 20-30%, which is among the highest in foodborne diseases. For cooked and ready-to-eat foods, there is a zero-tolerance for *L. monocytogenes*. Currently, the FDA and USDA standard methods for detection of *L. monocytogenes* in food involve selective enrichment and isolation on selective media, followed by confirming the suspicious bacterial colonies by biochemical tests (Yousef and Carlstrom 2003). These procedures significantly improved the recovery of *L. monocytogenes* from foods and the confirmation method is quite specific. However, these procedures are time-consuming. The whole procedure takes approximately one week, while the meat products have very
limited shelf life. Therefore, novel approaches enabling sensitive and specific detection of spoilage microorganisms and pathogens within hours are greatly in demand.

Recently, real-time PCR has emerged as a powerful diagnostic tool in both medical and agricultural fields. Specific detection of microorganisms with a reported sensitivity of 1CFU/ml can be achieved within hours (Chen et al., 1997; Sharma and Carlson 2000; Davis et al., 2003; Perandin et al., 2004). In fluorogenic probe-based Taqman PCR, a dual-fluorescent labeled probe is included in the polymerase chain reaction (PCR) mixture. The intensity of the fluorescent signal generated is proportional to the amount of PCR products accumulated. The real-time PCR apparatus contains an optical module attached to the thermocycler, therefore the amplification results can be displayed almost in real-time on the connected computer screen. Due to the potential interference to the PCR reactions by the complex microbial background and food matrices, detection methods for food analysis need to be validated for detection specificity, sensitivity, and applicability in targeted foods.

Rapid detection of the targeted bacteria in the food products enables the industry to make proper decisions to handle the contaminated raw materials or final products on a timely basis. However, despite all the efforts to control microbial activities in the food system, foodborne illnesses and product spoilage still occur. Some of the pathogenic and spoilage microorganisms tend to be persistent in the food environment. These microorganisms become a major source for the secondary contamination of food (Møretrø and Langsrud, 2004). Our current knowledge on microorganisms is mainly gained from studying bacteria in liquid culture, i.e., planktonic cells. However, recent studies have shown that in nature, when microorganisms attach to surfaces, they often
form sessile structures called biofilms. Biofilm cells are significantly different from planktonic ones, particularly in that they are more resistant to adverse environments, including sanitation and processing treatments (Stewart et al., 2000; Gilbert et al., 2002).

In order to minimize food contamination by pathogenic or spoilage bacteria, it is critical to understand microbial activities of foodborne microorganisms, especially their biofilm formation in the food processing environments.

Most of our current knowledge regarding microbial biofilms is gained from studying single culture biofilms formed by “problematic” microorganisms. However, as microbial consortia, biofilms in natural or processing environments often involve multiple organisms. Studies on single culture biofilms by commensal organisms are very limited; the contribution of commensals to the persistent of pathogenic or spoilage microbes in the mixed culture food ecosystems is much less understood. Knowledge regarding the initiation, development, and detachment of mixed culture ecosystem and the interactions among pathogenic, spoilage, commensal, and even beneficial organisms within such ecosystems is greatly in need.

An essential requirement to examine mixed culture ecosystem is to be able to determine microbial identity in the complicated ecosystems. Recent advancements in microbial identification using conserved ribosomal rRNA gene-encoding sequences and other related approaches make efficient investigation of the microbial composition in the ecosystems possible.
References


CHAPTER 2

LITERATURE REVIEW

Food is rich in nutrients, thus even low numbers of initial microbial contamination could result in rapid growth of spoilage or pathogenic organisms under optimal conditions. Both the food ingredients and the diversified microbial background in the ecosystems can interfere with the detection results. Therefore appropriate methods enabling rapid, specific and sensitive detection of the targeted microbes only, from the complicated food ecosystems, are greatly in need.

2.1 Conventional detection methods for specific microorganisms in foods

Conventional microbial detection methods rely on observing the physical growth of bacterial cells or monitoring the release of their metabolites. To isolate and identify the target organisms from the complicated food ecosystems, an enrichment procedure is commonly needed in microbial analysis of food samples. This is often followed by a subculturing step by plating the enrichment mixture on a selective or differential solid medium and incubating the samples in selective temperatures or atmospheres (Mossel 1977; Ray 2001; Yousef and Carlstrom 2003). Biochemical tests are conducted to
ultimately identify the microorganisms. Various biochemical tests are available to measure specific microbial metabolic activities, such as the catalase reaction, β-hemolysis, and H₂S release from triple sugar iron agar (Yousef and Carlstrom 2003). Customized API biochemical identification systems (bioMerieux, Durham, NC) are available to confirm a variety of organisms. These systems use strips that generally contain 20 miniature biochemical tests. Bacterial cultures are inoculated into test strips followed by 4 h to 72 h incubation. Currently, there are 16 identification kits covering most bacterial groups and more than 550 species (http://industry.biomerieux-usa.com/industry/food/api). Although conventional methods can be sensitive, they are very laborious and time consuming. Usually it takes anywhere from 48 h to a couple of weeks for results delivery.

2.2 Immunoassay

Immunoassays are based on the specific bindings between antigens and antibodies. Historically, they were used mainly in clinical diagnostics, but are becoming popular in rapid detection of microorganisms from food samples. Antigens are proteins or carbohydrates, and can be part of the physical structure or the secretion of a bacterial cell. When exposed to antigens, human or animal host can produce specific antibody proteins that can recognize and interact with antigens. Both polyclonal and monoclonal antibodies have been used in immunoassays and the choice of antibodies used determines the specificity of the immunoassay (Forbes et al., 1998). Several types of immunoassays have been used in food sample analysis, including enzyme linked immunosorbent assay (ELISA), immunodiffusion tests, immunofluorescent microscopy, immunomagnetic
separation, immunoprecipitation, and particle agglutination. Currently, a couple of commercial immunoassay-based detection systems are available to detect toxins or microbes in the food system, for example, the ImmunocardSTAT! E. coli O157:H7 detection kit, the RIDASCREEN Staphylococcus enterotoxin detection kit, the Organon-Teknika Listeria monocytogenes detection kit, the Salmonella-Tek Salmonella detection kit, and the NOW E. coli O157 antigen detection kit (Park et al., 1994; Yousef and Carlstrom 2003).

Depending on the analytical method, the immunoassay could take several minutes to several days, commonly in the range of hours. The detection limit of immunoassay varies by the analytical method as well. For example, ELISA, a very typical immunoassay, can detect less than 1ng of toxin ml\(^{-1}\) or g\(^{-1}\) of food, and 10\(^2\) CFU to 10\(^5\) CFU of bacterial cells ml\(^{-1}\) of food samples (Park et al., 1994; Mattingly et al., 1988; de Boer and Beumer 1999; Kovas and Rasky 2000; Chapman et al., 2003; Pettipher et al., 2004). Another method, immunofluorescent microscopy was reported to have a detection level of 10\(^{3.5}\) CFU Salmonella or 10\(^{3.11}\) CFU Listeria ml\(^{-1}\) of samples (Cloak et al., 1999; Sheridan et al., 1997). An enrichment, for 16-24 h at least, is usually required for the detection of microorganisms in foods using immunoassays. Therefore, the whole testing procedure takes at least one day.

### 2.2.1 Enzyme-linked immunosorbent assay (ELISA)

In ELISAs, enzymes, which can catalyze a reaction generating a colored end product that can be examined by spectrophotometer, are usually labeled to the toxin- or microbe-specific antibodies. In this way, the reaction of antigens and antibodies can be
amplified through the catalytic activity by the enzyme and the detection sensitivity can be greatly increased. Solid-phase immunoassay is the most common format of ELISA: the antibody is fixed to the inside of the wells of a plastic plate in most cases. After the sample is added to the wells, the antigens in the sample will react with the fixed antibodies and form antibody-antigen complexes. The addition of a second antibody labeled with an enzyme, will allow the formation of a sandwich with the existing complex. After washing and the enzyme reacting with its substrate, the colored end product forms (Forbes et al., 1998).

ELISA usually takes 1 h for toxin and 20-36 hours for cells (Pettipher, 2004). Because of its accuracy and automation capability, most commercial immunoassay kits for food testing are based on ELISA. However, ELISA still has its limitations. For instance, without enrichment, the sensitivity of ELISA may not be enough to detect the microorganisms at very low level. Therefore, the time required for sample analysis including the pre-enrichment and/or enrichment procedures could be much longer than ELISA itself (de Boer and Beumer 1999, Chapman et al., 2003).

2.2.2 Immunodiffusion tests

Double immunodiffusion can detect soluble antigen. The diffusion of antibody and the soluble antigen leads them to meet and form a visible band. This technique could be used to detect the presence of microorganisms and enterotoxin in food samples but it takes 3-5 days and is too slow to be commonly used.

A modified version of double immunodiffusion applies an electrical current to speed up the diffusion of antigen and antibody. However, its sensitivity is about $10^3$
organisms/ml of fluid and large amount of sample is needed, thus it is also not very popular in routine food analysis (Moberg et al., 1988).

2.2.3 Immunofluorescent microscopy assay

Immunofluorescent microscopy assays are popular in clinical diagnostics because of their major advantage of visual assessment. Recently, immunofluorescent microscopy assay has been reported to detect Salmonella and L. monocytogenes in food samples and the detection sensitivity is around $10^3$ to $10^4$ CFU ml$^{-1}$ in non-enriched samples (Sheridan et al., 1997; Cloak et al., 1999).

2.2.4 Immunomagnetic separation (IMS)

IMS uses antibodies coupled to magnetic particles to capture antigens of the microorganisms from food samples or pre-enrichment media (Skjerva et al., 1990). IMS has been used in the detection of L. monocytogenes in foods by conventional methods, in the detection of Salmonella by PCR, and in the detection of E. coli followed by ELISA. The addition of IMS can result an increase in detection sensitivity approximately 100-fold above that of ELISA (Skjerva et al., 1990; Rijpens et al., 1999; Tsai et al., 2000). The selectively captured antigens and microorganisms are then plated or further tested using other assays. Additionally, IMS is a very good supplementary method for other assays, but it is a labor intensive technology.
2.2.5 Immunoprecipitation assay

Immunoprecipitation assay has been used for detecting \textit{E.coli} O157:H7, \textit{Listeria}, \textit{Vibrio} and \textit{Salmonella} in food samples. Immunoprecipitation assay is based on the technology similar to home pregnancy tests: basically, the detection antibody is labeled by colloidal gold and the sample is loaded to the chambers to get results; no washing or manipulation is required and the assays can be completed within 10 minutes after sample enrichment (Feldsine \textit{et al.}, 1997). The assay itself is simple and very quick but long pre-enrichment is needed for food sample analysis (Dziezak 1987; Feng 2001).

2.2.6 Particle agglutination

Latex agglutination (LA) and reverse passive latex agglutination (RPLA) are two members of particle agglutination. LA has been used to quickly identify serologically pure bacterial culture isolates from food samples (D’oust \textit{et al.}, 1991; Feng 1997; Siragusa \textit{et al.}, 2004). Reverse passive latex agglutination is a modified format of LA testing for soluble antigens. It is used mostly in testing for toxins in food extracts or for toxin produced by pure bacterial cultures isolated from food, for example, the enterotoxin of \textit{C. perfringens} (Feng 1997; Feng 2001). The limitation of both LA and RPLA is that pure bacterial culture isolates are needed before the conduction of immunoassays.

2.3 Nucleic acid probe based techniques

Nucleic acid probe-based techniques use labeled gene probes to detect and identify specific microorganisms by hybridization. The DNA fragments are labeled as
probes so that one may determine whether they could form hybrids with the nucleic acid extracted from a sample. The nucleic acid (DNA or RNA) targeted and hybridized by the probe could be from chromosomes, plasmids, mitochondria or ribosome in the microorganisms. Nucleic acid probe-based techniques have been reported in detection and identification of bacteria and viruses in clinical, food and environmental samples, and choosing a unique fragment from other microorganisms as target nucleic acid is the key to the specificity of these techniques (Datta et al., 1987; King et al., 1989; Datta et al., 1990; Charteris et al., 1997; Willshaw et al., 1993; Alexander et al., 2001). Commercial DNA probe kits are also available for testing *Listeria, E. coli, Salmonella, Campylobacter, Staphylococcus aureus* and *Yersinia enterocolitica et al.* in food system, such as the Gene-Trak *Salmonella* assay, the Gene-Trak *E. coli* assay, and the Accuprobe *Listeria* assay (Feng 2001). For the detection of pathogens, virulence genes are frequently used as targets for probe development because they are typically conserved among a specific group of microorganisms but absent or with different sequences in other microorganisms (Batt 1999). The sensitivity of nucleic acid probe-based techniques is about $10^4$-$10^6$ gene copies (Petippher et al., 2004). Because it has multiple copies in one bacterial cell and is considered a distinct signature of each bacterium, the 16S rRNA-encoding gene has been widely used as a target nucleic acid in these techniques with improved detection sensitivity. However, achieving specificity in the assays using probes targeting 16S rRNA-encoding gene could be challenging because of the possibility of non-stringent hybridization (Kalamaki et al., 1996; Swaminathan and Feng 1994).
2.4 Conventional polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is amplification of specific nucleic acid fragment by a thermocycler at the presence of a pair of primers, a template, dNTPs, and the thermoresistant DNA polymerase. It has been used extensively for more than twenty years to rapidly detect, characterize, and identify a variety of organisms by detecting the presence of target gene fragment (Campbell 1996).

In a conventional PCR setting, a nucleic acid sequence is targeted and amplified with the enzymatic amplification function of a thermostable DNA polymerase and two flanking oligonucleotide primers. The most frequently used DNA polymerase is Taq polymerase, which was originally purified from *Thermus aquaticus* and can now also be obtained from recombinant *E. coli*. Primers are commonly 15-25 bp long with GC content above 40% and are designed to anneal to the unique fragments containing the complementing target nucleic acid sequences. For the amplification of the target, PCR usually involves 30-40 cycles of denaturation of DNA, annealing (hybridization of PCR primers to denatured DNA), and elongation. Since the amplification is exponential, one gene copy can be amplified to be approximately $10^9$ copies after 30 cycles of PCR amplification (Fig. 2.1).

To confirm the presence of the microorganisms in a sample, post-amplification analysis is required in addition to conventional PCR amplification, which usually involves fractionation of PCR products by agarose gel electrophoresis and staining with ethidium bromide (EB). The size of the amplicon is determined by comparing its migration in the gel to the migration of molecular weight standards. Sometimes, a
confirmation step, such as southern-blot analysis, is further needed to distinguish the real
amplicons from false positive amplification products (Cockerill 2002).

Conventional PCR allows *in vitro* amplification of a specific gene fragment. It
can be a powerful tool for analysis. However, certain shortcomings limit its effectiveness
in many cases. Non-specific amplification leading to false positive results is a major
problem associated with PCR, particularly non-specific products under low-stringency
conditions and primer dimers (Miller *et al.*, 1996). Post-amplification analysis is another
problem associated with PCR. Additional labor is required and carry-over contamination
of PCR products could also happen (Fratamico and Bagi, 2001; Hanna *et al.*, 2005).

2.5 Real-time PCR

Recently, significant improvements have been made in PCR applications. The
development of real-time PCR is one of the major advancements. Real-time PCR adds an
optical module to a conventional PCR assay, allowing real time detection of the
fluorescent signal during each replication cycle of the PCR. It permits rapid, dynamic
assessment of PCR products (amplicons), and lessens the possibility of contamination by
extraneous nucleic acids (Cockerill and Uhl 2002). Several commercial real-time PCR
testing platforms such as ABI PRISM 5700 and 7700(Applied Biosystems, Fosters City,
CA) and Icycler (Biorad, Hercules, CA), and Lightcycler (Roche Molecular
Biochemicals, Indianapolis, IN) have become available, which combine PCR
amplification and probe detection of target nucleic acids by incorporating a fluorescent
dye in the same closed reaction tube (Cockerill and Uhl 2002; Mackay 2004).
SYBRGreen, molecular beacon, Fluorescent resonance energy transfer (FRET) and Taqman assay are the major Real-time PCR formats.

2.5.1 SYBR Green real-time PCR

In SYBR Green real-time PCR, a highly specific, double-stranded DNA binding dye, SYBR Green I, is used to detect PCR product as it accumulates during PCR cycles. The amplification of gene fragments results in a net increase in fluorescence detected by the optical module. SYBR Green real-time PCR has been used for detection of foodborne microorganisms, such as *E.coli* O157:H7, *Salmonella*, *Staphylococcus aureus* and *Listeria monocytogenes* (Jothikumar and Griffiths 2002; Bhagwat 2003; Hein et al., 2001; Wang et al., 2004). SYBR green real time PCR is the cheapest one among all the real-time PCR assays, however, a specific product have to be determined by a melting curve analysis, which makes this assay not as discriminatory as other specific real-time chemistries (Wittwer *et al.*, 2001).

2.5.2 Molecular beacon

Molecular beacon real-time assay uses a single-stranded oligonucleotide probe that can form a stem-and-loop structure, and labeled with a fluorophore at the end of one arm and a quencher at the end of the other (Tyagi and Kramer 1996). The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. When molecular beacon is free in solution and maintains the form of stem-and-loop, it does not fluoresce. However, when it hybridizes its target sequence it undergoes a
conformational change and fluoresces brightly (Tyagi and Kramer 1996; Tyagi et al., 1998) (Fig. 2.2). Molecular beacon real-time PCR assay offers confirmation of target nucleic acid and capability of multiplexing, it has been used on specific detection of microorganisms in food systems, such as *E.coli* in milk, *Salmonella* in fruits and vegetables, *Bacillus spp.* in milk (McKillip and Drake 2000; Liming and Bhagwat 2004; Gore et al., 2003). Molecular beacon real-time PCR has its advantages such as high specificity; however, it has the highest costs among real-time PCR assays and may be too expensive to be used extendedly in food systems (Mckillip and Drake 2004).

### 2.5.3 Fluorescent resonance energy transfer (FRET)

Two fluorescent-labeled probes are used to anneal the target sequence in a head-to-tail arrangement. One probe is usually labeled with fluorescein; the second probe is labeled with a dye with a longer emission wavelength such as LightCycler’s Red 640. The energy transfer is depending on the space between the first dye and second dye, when the two probes are annealed to the target sequence, the light source will excite the first dye to emit light and the light is closed enough to excite the second dye to emit light at the wavelength collected by the instruments; while these two probes are separate, energy transfer will not happen. FRET is popularly used by Lightcycler owners in studying bacteria, parasites and viruses. Melting curve can be performed to identify the mutation or to differentiate different species in the same genus. However, in some tests melting curve needs to be performed to differentiate the products of negative controls showing amplification from the true positive results. And also, multiplex reactions are barely reported (Cockerill and Uhl 2002).
2.6 Taqman Real-time PCR

Among the various real-time PCR formats, Taqman® real-time PCR, incorporates the 5’ nuclease chemistry and includes a probe labeled with 5’ fluorescent reporter dye and 3’ quenching dye for signal detection, making it a powerful diagnostic tool for bacteria and viruses in medical and agricultural fields (Batt 1997). Because the signal detection in the Taqman® system depends on both the annealing of primers to complementing sequences for PCR amplicon synthesis and the hybridization between complementing sequence of the oligonucleotide probe and the PCR amplicons, the false positive rate of the detection is significantly reduced.

In Taqman® real-time PCR, the 5’ nuclease chemistry and a third oligonucleotide probe (Fig. 2.3), labeled with 5’ fluorescent reporter dye and 3’ quenching dye for signal detection, in addition to the two primers in routine PCR, are incorporated. As shown in Fig. 2.3, in the beginning of one cycle, primers and probe are annealed to the target DNA. At this time, the emission of reporter dye in the integrate probe is quenched by the quencher dye nearby; when the amplification carries on, the probe is cleaved by the 5’ nuclease function of the Taqman polymerase, therefore the quencher dye is too distant to quench the emission of the reporter dye, and the signals from the reporter dye can be captured by the optical module.

Because the signal detection in the Taqman® system depends on both the annealing of primers to complementatory sequences for PCR amplicon synthesis and the hybridization between complementory sequence of the oligonucleotide probe and the PCR amplicons, the false positive rate of the detection is significantly reduced. Compared
to methods from culture-based to conventional PCR and other two real-time PCR formats, Taqman real-time PCR has the following characteristics:

1. High specificity with decreased risk for specimen or amplicon contamination compared to conventional PCR, and increased discrimination compared to SYBR Green real-time PCR.

2. High sensitivity, similar to conventional PCR.

3. Rapid. The detection can be completed within a few hours. It has the potential to replace conventional PCR assays, as well as standard antigen or work intense procedures for culture-based assays.

4. Capable of multiplex reactions detecting multiple genes from one organism or multiple organisms, simultaneously.

5. With medium price, cheaper than molecular beacon real-time PCR.

Melting point (Tm) of dsDNA is the temperature that 50% of the DNA is single stranded. The length and G+C content of the DNA determine Tm. Some researchers prefer to use SYBR green and FRET real-time PCR because they can use melting curve analysis to detect single mutation and differentiate amplicons with tiny differences. No melting curve analysis is for Taqman real-time PCR because the probes are cleaved by Taq polymerase. Recently, Housni et al. (Housni et al., 2003) reported to use a different procedure to avoid the hydrolysis of the Taqman probe and in this way melting analysis was performed for single-nucleotide polymorphism genotyping.

Taqman assay has emerged as a powerful diagnostic tool in both medical and agricultural fields. So far, Taqman real-time PCR has reportedly been used in the detection of *Escherichia coli* O157:H7 in dairy wastewater wetland, *E. coli* isolates in the
environment, as well as in routine clinical diagnosis of malaria parasites, *Plasmodium falciparum, Plasmodium vivax*, and *Plasmodium ovale* and many other bacteria and viruses in environmental and clinical samples (Ibekwe *et al.*, 2002; Lee *et al.*, 2002; Perandin *et al.*, 2004, Pinzani *et al.*, 2004; Volkmann *et al.*, 2004). Detection level from 1-60 of bacteria or viruses gene copies per PCR reaction was reported within several hours.

The successful applications of Taqman real-time PCR in the clinical diagnostic field have demonstrated that this novel technology is a powerful molecular diagnostic tool. However, its application in food systems is relatively limited (Chen *et al.*, 1997; Cox *et al.*, 1998; Nogva *et al.*, 2000; Sharma and Carlson 2000, Wu *et al.*, 2004). Food often contains ingredients and additives that may interfere with rapid detection procedures. Therefore, the applicability of the real-time PCR systems needs to be verified and optimized for individual food commodities. With an efficient and rapid detection system for pathogenic and spoilage microorganisms in food samples, the chance of releasing health hazardous food products can be minimized to ensure food safety. Particularly, real-time PCR provides the proper quality assurance for products with minimum processing.

### 2.7 *Alicyclobacillus* sp. and juice spoilage

In 1992, three previously classified *Bacillus* thermoacidophiles (*B. acidocaldarius, B. acidoterrestris*, and *B. cycloheptanicus*) were reassigned to *A. acidocaldarius, A. acidoterrestris*, and *A. cycloheptanicus* within *Alicyclobacillus*, which is a genus distinctive from *Bacillus* as shown by 16S rDNA sequences analysis, and by uniquely
containing ω-alicyclic fatty acid as the major membranous lipid component (Wisotzkey et al., 1992). From then on, 7 new species including *A. hesperidum* and *Alicyclobacillus* genomic species 1 and 2 (Albuquerque et al., 2000; Goto et al., 2002a), *A. acidiphilus* (Matsubara et al., 2002), *A. herbarius* (Goto et al., 2002b), *A. sendaiensis* (Tsuruoka et al., 2002), and *A. pomorum* (Goto et al., 2003) have been isolated from different places including soil and acidic environments and added to this genus.

*Alicyclobacillus* spp. are thermophilic and aciduric Gram-positive, spore-forming, rod-shaped bacteria. These microorganisms grow at relatively high temperature and low pH (Darland and Brock, 1971; Rainey et al., 1994); and some strains from *A. acidocaldarius* and *A. acidoterrestris* have been recognized as spoilage agents in acid foods, particularly in fruit juices. These spoilage organisms cause a flat sour type of spoilage and produce offensive-smelling compounds including guaiacol (o-methoxy phenol) and other taint chemicals, like 2, 6-dibromophenol and 2,6-dichlorophenol (Pettipher et al., 1997; Orr et al., 2000; Jensen and Whitfield, 2003) in juice products and their presence is becoming a major concern for the food industry (Cerny et al., 1984; Yamazaki et al., 1996; Komitopoulou et al., 1999). These organisms all have the ability to form spores enable them to survive heat treatment processes, such as pasteurization in fruit juice production, and the ability to grow in acidic environment though they may vary in growth limit and spore thermal resistance strain-to-strain. A D-value, at 90°C of 13 to 22 minutes for *Alicyclobacillus* spores in orange and apple juice (Previdi et al., 1997) indicates that higher temperature treatments than the current processing procedures in juice industry are needed to kill all microorganisms including spores and vegetative cells; however, the extreme treatment is not applicable because of its possible detrimental
effects to the quality of juice products caused by volatilization of flavor compounds and creation of off-flavors by catalysis of chemical reactions (Kuntz 1994; Connor 2004). In addition to apple juices, growth of Alicyclobacillus vegetative cells and spore germination in orange juice, grapefruit juice, white grape juice, tomato juice, and pear juice have been reported (Pettipher et al., 1997, Evancho and Walls, 2001, Silva and Gibbs, 2001). Therefore, spoilage Alicyclobacillus sp. may be present in fresh squeezed, pasteurized, or hot-filled juices as spores or vegetative cells. If appropriate amounts of Alicyclobacillus sp. are present in the product after processing, particularly in warmer climates, shelf-stable juices may develop the off-flavors within the first month of storage. The significant reduction of the quality of the juice products will subsequently lower the consumer satisfaction (Pettipher et al., 1997; Jensen and Whitfield 2003; Connor 2004).

2.8 Detection of Alicyclobacillus

Current detection methods for Alicyclobacillus from food products are mainly plate-based methods. They start with a heat treatment to juice or juice concentrate samples followed by plating on K-agar, orange serum agar (OSA), acidified potato dextrose agar (acidified PDA), pH 4.0 malt extract agar, or Bacillus acidocaldarius medium (BAM) agar with the sample or the diluted samples and incubation in 37-50°C for 3-7 days (Previdi et al., 1997; Pinhatti et al., 1997). These methods activate most of the spores in the food samples and can provide accurate results, but take too long to complete. A new Alicyclobacillus detection system called the BioSys identification system (BioSys, Ann Arbor, MI) becomes commercially available recently. It takes 36 h for proper detection of Alicyclobacillus sp. Gas chromatography along with mass
spectrometer (GC-MS) was also reported to detect guaiacol in the food sample and indicate the presence of *Alicyclobacillus* cells. However, this method is not applicable to the samples that are already contaminated but not yet developed the off-smell compound. Therefore, a more rapid detection method that can provide accurate results as plate-based methods, but within hours, is preferred.

### 2.9 Squalene-hopene-catalyse (SHC) in *Alicyclobacillus*

SHC is an enzyme catalyzing the cyclization of squalene to hopanoids, a class of triterpenoid lipids, important membrane components of *Alicyclobacillus* sp. involved in maintaining membrane fluidity and stability and therefore is critical for the biological functionality of the organisms at extreme environmental conditions (Kannenberg and Poralla, 1999). SHC have been found in some Gram-positive and Gram-negative bacteria. The amino acid sequence comparison showed that *A. acidocaldarius* and *A. acidoterrestris* have a high similarity and their sequences are unique from other microorganisms, including Gram-positive *Bacillus*, Gram-negative *Zymomonas mobilis* and *Bradyrhizobium japonicum* (Perzl et al., 1997). The crystal structure and the function of SHC from *A. acidocaldarius* were well studied by Wendt *et al.* (1999). The catalytic mechanism of SHC is closely related to 2, 3-Oxidosqualen cyclase (OS-cyclase) in eubacteri. However, information about *Alicyclobacillus* SHC gene sequence in the DNA database is limited and insufficient for the primer-and-probe development.
2.10 *Listeria monocytogenes* and food safety

*Listeria monocytogenes* is a foodborne pathogen causing listeriosis mainly in high-risk groups, including pregnant women, neonates, and immunocompromised adults (Slutsker and Schuchat 1999). The outbreaks of human listeriosis were reported in many countries since 1980s and involved various kinds of foods, including milk, cheese, vegetables, coleslaw, seafood, raw meat and ready-to-eat meat (Ryser 1999). Listeriosis symptoms are variable and depend on the individual's susceptibility. Symptoms may be limited to fever, fatigue, nausea, vomiting and diarrhea; however, these symptoms can precede a more serious illness: the more serious forms of listeriosis can result in meningitis (brain infections) and septicemia (bacteria in the bloodstream). Pregnant women may contract flu-like symptoms of listeriosis; complications can result in miscarriage, stillbirth, or septicemia or meningitis in the newborn (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_g.htm#symptoms). Every year there are about 5000 cases of listeriosis, which has a mortality rate of approximately 20% to 30% (Gellen et al., 1999).

The high-case fatality rate and increasing outbreaks resulted in increased attention to *L. monocytogenes*. It is a gram-positive rod and was reported to have an optimal growth temperature between 30 and 37°C and a highest growth temperature at 45°C, optimal a\textsubscript{w} about 0.97, optimal pH from neutral to slightly alkaline (Lou and Yousef 1999). However, *L. monocytogenes* is a psychrotrophic bacterium and resists to heat, salt, nitrite and acidity much better than many organisms (http://vm.cfsan.fda.gov/~mow/fsislist.html). The lowest growth temperature of *L. monocytogenes* was reported to be close to 0°C (Hudson et al., 1994) in an appropriate
medium. It was also reported to be an acid-tolerant bacterium and can grow in water activity as low as 0.9 (Reimer et al., 1988). The above characteristics indicate \textit{L. monocytogenes} can survive or multiply in various food products even in refrigeration temperature, which makes it very dangerous in ready-to-eat foods.

2.11 Detection of \textit{Listeria monocytogenes}

Due to the potential risk of this bacterium, according to the USDA (http://vm.cfsan.fda.gov/~mow/fsislist.html), neither the Food Safety and Inspection Service (FSIS) nor FDA will accept cooked, ready-to-eat foods with any detectable \textit{L. monocytogenes}. This is called "zero tolerance" for the bacterium. Therefore, a sensitive and specific detection method is highly in need. Currently, the standard methods of FDA and USDA use selective medium to enrich and isolate \textit{L. monocytogenes} from food samples, and finally the suspicious bacterial colonies are confirmed by biochemical tests (Yousef and Carlstrom 2003). These methods used the unique characteristics of \textit{L. monocytogenes} to selectively enrich and isolate \textit{L. monocytogenes} from other bacteria and thus highly improved the specificity and sensitivity of the detection of \textit{L. monocytogenes} from various foods. However, these methods are still very time-consuming, the whole procedure taking approximately one week.

In order to shorten the time needed to fulfill the requirement of a rapid, sensitive and specific detection method of \textit{L. monocytogenes} from food samples, efforts to apply novel techniques including ELISA, nucleic-acid probe hybridization and regular PCR have never stopped. However, the sensitivity of ELISA and nucleic-acid probe
hybridization is still low, the regular PCR obtained great sensitivity but the specificity is not ideal because of non-stringent amplification.

2.12 The hly and prfA genes in *Listeria monocytogenes*

As other pathogens, *L. monocytogenes* has its unique virulence genes. The *hly* gene encodes listeriolysin O, a secreted protein of 58-60KD belonging to a family of pore-forming sulfhydryl cytolysin causing hemolysis. It is believed that listeriolysin O represent a major virulence determinant. A clear correlation between hemolysis and virulence has been clearly established, all virulent strains of *L. monocytogenes* have been reported to be hemolytic and non-hemolytic strains are avirulent (Gaillard *et al.*, 1986; Katharious *et al.*, 1987). As a regulatory factor of several virulence genes, the *prfA* encoding protein plays an important role in determine the virulence of *L. monocytogenes*.

2.13 Microbial biofilms in the food system

Certain bacteria can attach to various surfaces, such as metal, plastic, medical implant materials and tissue, and form a complex, multicellular structure called a biofilm (Costerson and Lappin-Scott, 1995). Biofilm is generally considered problematic. The positive function of cell immobilization through biofilm formation in beer and lactic acid fermentation process is an exception (Cotton *et al.*, 2001). The major concern about the biofilms in the food industry is the detriments caused by unintentionally formed biofilms on various surfaces from processing equipment to raw food materials (Wirtanen *et al.*, 2000). Persistent pathogen contaminations were found closely related to the presence of
these pathogens in the form of biofilms in the processing environments. Conventional methods of killing planktonic cells are often ineffective against biofilm bacteria. Therefore, biofilms are important sources of secondary contamination of spoilage and pathogenic organisms (Hood and Zottola, 1995; Møretrø and Langsrud, 2004).

Several mechanisms might have contributed to the increased resistance of biofilm microbes to antibiotics, chlorine and detergents, including the non-invasive biofilm structure, altered gene expression to activate resistance, lowered growth rate and acquisition of resistance-encoding genes by horizontal gene transfer (Gilbert et al., 2002). In fact, biofilm could be one of the major persistent mechanisms of microbial contamination in the food system.

2.14 Biofilm Characteristics

Fig.2.4 illustrated the single-culture biofilm development model by *Pseudomonas aeruginosa*. Biofilm formation includes several stages from initiation, maturation to detachment (O’Toole et al., 2000). The process initiates from planktonic cells forming cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. At the presence of developmental signals, microcolonies extend to form mature, three-dimensional biofilm architecture. To complete the cycle of biofilm development, some cells detach from the biofilm, return to a planktonic lifestyle and may start another circle of biofilm formation (O’Toole et al., 2000). The development of biofilm varies by the bacterial strains, surface, temperature, nutrients, and the availability of other bacteria in the environment (Costerson and Lappin-Scott 1995). While biofilm may derive from
planktonic cells from single strain bacterial culture attaching to the surface, mixed culture biofilms involving multiple organisms are the most prevalent forms in the natural environment.

2.14.1 Single culture biofilm

Some microorganisms naturally have a higher tendency to produce biofilm than others in specific environment (Watnick and Kolter 2000). It is found that in several bacteria species including *E. coli* and *Vibrio cholerae*, type IV pili and flagella can accelerate the initial attachment to the surface for the development of single-culture biofilm (Pratt and Kolter 1998; Watnick *et al.*, 1999). It has been clearly demonstrated that the quorum-sensing molecules acyl-homoserine lactones (acyl-HSLs) are important for the development of single-culture biofilm by *P. aureginosa* (Stickler *et al.*, 1998; Davies *et al.*, 1998). Furthermore, the expression of certain genes in sessile cells differs from that in planktonic ones. For example, the synthesis of flagellin is decreased and the production of exopolysacchride and some genes related to resistance are increased in sessile cells (Prigent-Combaret *et al*., 1999; Whiteley *et al*., 2000). Since biofilm cells are more resistant to the sanitation and antibiotics than planktonic cells, a better biofilm forming ability of pathogens and spoilage microorganisms may correlate to the tendency of persistence in food systems. In addition, a relationship between the virulence and biofilm-forming ability of foodborne pathogens was suggested in the past (Deighton and Balkau 1990). However, studies on biofilm formation by foodborne microorganisms are so far limited. Some of the reports using different experimental approaches even gave contradict results. For example, Djordjevic *et al.* (2002) reported lineage I
*L. monocytogenes* strains containing major strains from human listeriosis cases produced significantly greater biofilms than lineage II and III strains in a rapid microtiter plate assay, and their results suggested a potential correlation between biofilm-forming and virulence. However, Kalmokoff *et al.* (2001) reported that there was not such a correlation. Different temperatures, time points, nutrients, and surfaces might have contributed to the diversified results in these studies, and the lack of standard methods certainly did not help with the situation (Marsh *et al.*, 2003). Proper methods to evaluate biofilm formation are essential in such studies. Marsh *et al* (2003) compared biofilm formation by several *L. monocytogenes* strains associated with outbreaks and consistent results were obtained using three different approaches. The results suggested the effectiveness of such methodology in evaluating the strain’s ability in forming biofilm.

### 2.14.2 Mixed culture biofilm

Bacteria in nature often live in multispecies communities bound to surfaces under changing conditions (Holah and Gibson 2000; Bagge-Ravn *et al.*, 2003). Recent studies showed that microbial biofilm communities could be viewed as aggregates of a number of different organisms in a consortium with mutual benefits (Kolenbrander 2000). For instance, the dental plaque can be viewed as a complex biofilm ecosystem formed by any different microorganisms. More than 500 bacterial taxa have been isolated from oral surfaces (Kolenbrander 2000). In food processing environment, multiple bacterial taxa have also been isolated as well. Because of the possible competition and cooperation among the microorganisms, the behavior of bacteria in mixed-culture could be significantly different from that in the single-culture (Cook *et al.*, 1998).
For example, various types of cell-to-cell signals might be found in an ecosystem, their effects on bacterial neighbors could vary from beneficial, detrimental, to even fatal (Moller et al., 1998). The interactions among microbes in the biofilm may directly determine the fate of the microorganisms involved (Watnick and Kolter 2000). Studies on mixed-culture biofilms often involve identification of the organisms in the ecosystem and examination of the interactions among these microbes in the consortium. It has been found coaggregation and conjugation among certain strains could facilitate biofilm development (Kolenbrander 1988; Ghigo 2001). The presence of aerobic bacteria could facilitate biofilm development by anaerobic bacteria (Bradshaw et al., 1997). Certain bacterial strains could affect the behaviors of others (Cook et al., 1998; Leriche and Carpentier 2000; Al-Bakri et al., 2004). However, so far most of the mixed culture biofilm studies were conducted in dental or water environments; data from food systems are very limited. In order to develop effective industrial strategies to minimize the persistent microbial contamination in food industry, a better understanding of mixed culture biofilm formed by foodborne microorganisms is essential and may be even more important than understanding of single-culture biofilm.

2.15 Research tools to examine microbial biofilms

2.15.1 Detection of the presence of microorganisms in biofilm

16S rRNA gene sequence analysis has been widely used to identify the microbial composition in the ecosystem. Most of the bacteria have multiple copies of the small ribosomal subunit RNA (16S rRNA) gene, also called 16S rDNA, which are highly conserved, share universal function, and widely distributed among bacteria (Corless et al.,
16S rRNA is a structural backbone of the ribosomal small subunit, and also works as an interaction site between the small and large subunits. 16S rRNA has a complicated looped secondary structure containing many helical regions, and it also has a critical binding site involved in the initiation of protein synthesis and may have function in decoding and elongation in protein synthesis (Brimacombe 1988). Because of the high conservation in all organisms and variation contained within the gene, 16S rDNA has been used extensively in determining taxonomic classifications and evolutionary relationships (Brimacombe 1988; Weisburg et al., 1991). The 16S rDNA is approximately 1500 bp and was first sequenced from *E. coli* in 1971, and since then 16S rDNA has been used as a good target for taxa identification and rapid detection systems because of the conservation and variation regions contained within the gene (Connor 2004). Analysis of the sequences of 16S rDNA and the spacer region between 16S rDNA and 23S rDNA further assists differentiation of bacteria, from genus to species.

In addition, fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR), real-time PCR, and biochemical methods including API tests have been used for the identification of unknown bacterial species in natural mixed-culture biofilm (Tanner *et al.*, 1994; Dewhirst *et al.*, 2000; Aoi *et al.*, 2004).

### 2.15.2 Visualization of the development of biofilm structure

Observation of the composition and distribution of microorganisms involved in a biofilm is essential to understand the function of biofilm. With the aid of various microscopy techniques, including light microscopy, confocal scanning laser microscopy (CSLM), scanning probe microscopy (SPM), scanning electron microscopy (SEM),
transmission electron microscopy (TEM) and environmental electron microscopy (ESEM), we can visualize the biofilm when it is dehydrated, partly dehydrated or hydrated.

2.15.2.1 Light microscopy

The most common light microscopic techniques used in biofilm studies are bright-field-, dark-field-, phase-contrast- and fluorescence microscopic techniques. The light types are different among the above four techniques; for example, with the fluorescence microscopic technique, the light is emitted by the excited object instead of absorbed in other techniques. Light microscopy can be used to examine fully hydrated biofilms. However, the sample can only be a few micrometers thick. In order to visualize the 3D structure of biofilms, more advanced techniques, such as CSLM, are necessary.

2.15.2.2 Confocal scanning laser microscopy (CSLM)

CSLM uses a computer-controlled motor to move the microscope up and down to focus on different Z-planes in the thick sample (Lawrence et al., 1991). In a focal Z plane, the x-y plane information of the "confocal spot" is largely obstructed by the pinhole with the significantly reduced information above or below the focal Z plane (Davey and O’toole, 2000). By combining the x-y information in different focal Z planes, CSLM allows a 3-D noninvasive visualization of cells in the mature biofilms without damaging the biofilm structures.
2.15.2.3 Scanning probe microscopy (SPM)

Atomic force microscopy (AFM) is the major format of SPM. It uses a sharp physical probe to give information on the surface of the sample at high resolution by sensing the force between the probe and the surface, and the position of the probe and the feedback signal are recorded electronically to produce a three dimensional picture of the surface or a line profile with height measurements (Lal and John 1994). Partly dehydrated biofilm can be analyzed with AFM.

2.15.2.4 Electron microscopy

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and Environmental electron microscopy (ESEM) have played important roles in biofilm studies (Knutton 1995). By bombarding the material surface with a beam of electrons and detecting those emitted particles, SEM allows recording of high magnification of biofilm surface structures and imaging of shapes after proper fixation and dehydration of the sample. SEM enables a resolution of about 10 nanometers, giving details of the material’s structure. TEM allows visualization of internal structures of samples by passing the electron beam through the samples.

However, the requirements of SEM and TEM, such as a high vacuum to the maintenance of the electron gun and a thin metal coating to analyze an insulator, mean that dehydration and metal coating of the sample are necessary before observation, raising the risk of artifacts (Knutton 1995).

As an updated version of SEM, ESEM uses a system of differential pumping: the electron gun can be maintained at high vacuum while the sample chamber can be kept at...
a constant pressure (Knutton 1995). By using this system, partly dehydrated biofilm with less prior treatment can be examined by ESEM, permitting images with fewer artifacts than by SEM.

2.16 Horizontal gene transfer and biofilm

Recent studies showed that bacteria isolated from food samples carry multiple antibiotics resistance genes originated from other bacterial species (Perreten et al., 1997). Naturally existing biofilm may be considered as a microbial community with a gene pool composed of genomes from different organisms. Biofilm may provide an ideal environment for bacteria to acquire beneficial genes, such as those encoding antibiotics resistance, from other microorganisms in the same community, for better survival.

Conjugation, transformation, and transduction are three common forms of natural gene transfer. It has been reported that a biofilm environment is suitable for conjugation (Angles et al., 1994; Haunser and Wuertz 1998). It has also been demonstrated in E. coli, that conjugation served as an important mechanism for biofilm development, independent from quorum sensing (Ghigo 2001). Meanwhile, bacterial surface ligand-and-receptor interaction-mediated cell aggregation was found to be essential in oral bacteria biofilm assembly. Therefore, it is of particular interest to see whether there is a correlation between high frequency conjugation and biofilm formation in foodborne microorganisms, and if there is key surface factor(s) involved in the forming of these biofilms.
2.17 *Lactococcus lactis* in food industry

Lactococci are important fermentation starter cultures. As commensal organisms, they are widely distributed (Salama *et al.*, 1993; Heilig *et al.*, 2002) and can be found co-existing with many other organisms, including pathogens such as *L. monocytogenes*, in natural and food processing environments, most commonly in the form of mixed culture ecosystems. However, to date, information regarding lactococcal biofilm formation is very limited (Mercier *et al.*, 2002). The contribution of commensal organisms in mixed culture biofilm development involving pathogens and other risks associated with these biofilms have not been fully explored.

2.18 Gene transfer in *Lactococcus lactis*

Lactococci are susceptible to various gene transfer mechanisms (Gasson 1990). Many important traits, including lactose utilization, proteolytic system, bacterial phage resistance, and nisin production and immunity, are associated with mobile elements. Of particular interest, a cell-clumping associated high frequency conjugal gene transfer system has been reported in two similar settings involving *Lactococcus lactis* strains ML3 and 712. In both cases, the conjugative elements, being the sex factor in 712 and pRS01 in ML3, mobilized the transfer of the Lac plasmid to the recipient cells by forming plasmid co-integrates. Some of the conjugation progenies exhibited cell auto-aggregation. When these clumping cells served as donors in the second round of mating, they transferred the Lac plasmid $10^2$-$10^7$ times more efficiently than the original donor strain (Anderson *et al.*, 1984; Gasson and Davies 1980a; Walsh and McKay 1981).
Divalent ions were required for auto-aggregation, and proteinase treatments significantly decreased both cell clumping (Clu⁺) and high frequency conjugation (Wang et al., 1994). Strain MG1363 is a non-clumping, plasmid-cured derivative from strain 712 with the sex factor retained in the chromosome. A cluA gene, which encoded a putative cell surface protein containing the well-conserved hexapeptide LPXTGE, was cloned from the sex factor. Expression of the CluA through upstream fusion of a lactococcal heat-shock promoter in MG1363 partially restored cell aggregation (Gordon et al., 1994).
References


Figure 2.1. Exponential amplification of gene copy in PCR
(Modified from Campbell 1996)

Figure 2.2. Principle of molecular beacon probe
(Modified from Tyagi and Kramer 1996)
Figure 2.3 Polymerase and probe in Taqman real-time PCR
(Modified from Batt 1997 and
http://www.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf)

Figure 2.4. Model of the biofilm development of Pseudomonas aeruginosa
(Modified from O’Toole et al., 2000)
CHAPTER 3

DISSERTATION OBJECTIVES

A fluorogenic probe-based Taqman® assay was chosen as the platform to develop the rapid detection system for foodborne microbes. Our first objective was to develop a rapid method to detect the acid- and thermo-resistant spoilage bacteria *Alicyclobacillus* spp. in foods. This involved identifying a target gene, squalene-hopene cyclase-encoding gene, in spoilage *A. acidocaldarius* and *A. acidoterrestris*, developing a primer-and-probe set suitable for the real-time PCR system, examining specificity of the primer-and-probe set to the targeted bacteria, and finally assessing the sensitivity of the developed method in juice products. The second objective of this dissertation was to set up a rapid detection system for the foodborne pathogen, *Listeria monocytogenes*. This involved developing two primer-and-probe sets suitable for the real-time PCR system, targeting the virulence factor listeriolysin-encoding gene, *hly*, and the virulence regulator factor-encoding gene, *prfA*, in *L. monocytogenes*. The study also examined specificity of the primer-and-probe sets to the targeted bacteria.

Rapid detection of the contaminating bacteria in both the raw materials and final products is important for quality control in the food industry. Minimizing persistent contamination or secondary contamination due to the formation of microbial biofilms on the surfaces of both the raw materials and processing equipments is equally important.
Our third objective was to investigate the contribution of a surface protein, CluA, in *Lactococcus lactis* biofilm development. The gene *cluA* was cloned and expressed in *L. lactis*, and biofilm development by the transformants at the presence and absence of induced expression of CluA was compared. The risk of commensal organisms carrying intrinsic high frequency conjugation mechanism in dissemination of the drug-resistance encoding the broad-host range plasmid, pAMβ1, was also examined.

Even though certain strains cannot form biofilms by themselves, they might still be incorporated into a mixed-culture biofilm through interactions with partner organisms co-existing in the ecosystem. The fourth objective of this study was to evaluate the potential contribution of commensal organisms on the persistence of foodborne pathogens in the environment. Biofilm formations by single strain and mixed cultures were compared.
CHAPTER 4

A REAL-TIME POLYMERASE CHAIN REACTION-BASED METHOD FOR
RAPID AND SPECIFIC DETECTION OF SPOILAGE ALICYCLOBACILLUS SPP.
IN APPLE JUICE

4.1 Abstract

The aim of this project was to develop a real-time PCR-based rapid detection
method for spoilage Alicyclobacillus spp. in juice products.

The squalene-hopene cyclase-encoding gene was targeted for primer-and-probe
development. Gene fragments from representative strains were cloned, and PCR primers
and probe were designed by DNA sequence comparison. Selected bacteria were
examined for cross-reactivity by the new method. Cells were serially diluted in apple
juice and saline and examined by the new method to establish detection sensitivity.

Alicyclobacillus acidocaldarius and A. acidoterrestris were detected by the newly
developed Taqman® real-time PCR-based method without cross reactivity with other
common foodborne microorganisms. Detection of less than 10 cells per PCR reaction
from juice samples was accomplished within three to five hours.

This is the first reported real-time PCR-based detection method for
Alicyclobacillus spp. and its application in juice products is demonstrated. As a favorable
alternative for the laborious and time-consuming culture- or biochemical characterization-based techniques, the system has great potential for industrial applications from raw material screening to final product quality control.

4.2 Introduction

Food spoilage, as a consequence of growth of contaminating microorganisms or the release of their extracellular and intracellular enzymes, is causing significant financial losses to the food industry (Ray 2001). Applying extreme processing eliminates these microorganisms and inactivates the enzymes, but it can also significantly alter the physiochemical properties and nutritional values of treated food. Therefore proper screening of spoilage microorganisms in both raw materials and final products becomes very important in quality control, especially to alleviate unnecessary excessive processing. Conventional industrial practices for microbial detection from plate counting to biochemical analysis take anywhere from 48 hours to a couple of weeks. These methods are especially unsuitable for products with limited shelf life. Novel detection approaches enabling rapid and specific detection of spoilage microorganisms within hours are preferred.

The polymerase chain reaction (PCR) has been used extensively for years to rapidly amplify targeted DNA fragments. However, certain shortcomings, particularly high ratio of false positive results and the need for post-amplification analysis, limit its application in diagnostics and detection. By incorporating a fluorescent dye into the reaction mixture and coupling an optical module with the thermocycler, a real-time PCR setting enables almost real-time display of the amplification results on the computer.
screen. One of the advanced formats, Taqman® real-time PCR, incorporates the fluorogenic 5’ nuclease chemistry and includes a third oligonucleotide probe, labeled with 5’ fluorescent reporter dye and 3’ quenching dye, for signal detection (Bassler et al., 1995, Jaykus 2003). Because the signal detection in the Taqman® system depends on both the annealing of primers to complementing sequences for PCR amplicon synthesis and the hybridization between complementing sequence of the oligonucleotide probe and the PCR amplicons, the false positive rate of the detection is significantly reduced.

Fluorogenic probe-based Taqman® assay has emerged as a powerful diagnostic tool in both medical and agricultural fields (Batt 1997; Chen et al., 1997; Cox et al., 1998; Nogva et al., 2000; Sharma and Carlson 2000; Vishnubhatla et al., 2000; Ibekwe et al., 2002; Lee et al., 2002; Davis et al., 2003; Perandin et al., 2004, Pinzani et al., 2004; Volkmann et al., 2004, Wu et al., 2004). However, food often contains ingredients and additives that interfere with rapid detection procedures. Therefore, the applicability of the real-time PCR systems needs to be verified and optimized for individual food commodities.

*Alicyclobacillus acidocaldarius* and *A. acidoterrestris* are thermophilic and aciduric Gram-positive, spore-forming bacteria. They belong to the genus *Alicyclobacillus*, which is distinctive from *Bacillus* as shown by 16S rDNA sequences analysis, and by uniquely containing ω- alicyclic fatty acid as the major membranous lipid component (Wisotzkey et al., 1992). The presence of *Alicyclobacillus* spp. is becoming a major concern for the food industry. These organisms grow at relatively high temperature and low pH (Darland and Brock, 1971; Rainey et al., 1994), and some strains cause a flat sour type of spoilage and produce offensive-smelling compounds including guaiacol and
other taint chemicals (Pettipher et al., 1997; Orr et al., 2000; Jensen and Whitfield, 2003). Acid foods, particularly fruit juices, are susceptible to *Alicyclobacillus* spoilage (Cerny et al., 1984; Yamazaki et al., 1996; Komitopoulou et al., 1999). Current detection methods, including conventional culturing and biochemical analyses, take at least 2 days for results delivery, which do not meet industry’s expectation particularly for products with limited shelf life such as fruit juices. Therefore rapid, specific and sensitive detection approach for spoilage *Alicyclobacillus* spp. is of great interest to the industry.

The objective for this study was to develop the methodology for detection of spoilage *Alicyclobacillus* spp. in juice products using a Taqman®-based real-time PCR approach. This involves identifying a target gene in spoilage *A. acidocaldarius* and *A. acidoterrestris* and developing a primer-and-probe set suitable for the real-time PCR system. The study also examines the specificity of the primer-and-probe set to the targeted bacteria, and the sensitivity of the developed detection method in fruit juice.

4.3 Materials and methods

4.3.1 Bacterial strains and growth conditions

*Alicyclobacillus acidocaldarius* ATCC 43030 and *A. acidoterrestris* ATCC 49025 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown at 48°C in ATCC 573 broth (http://www.atcc.org/mediapdfs/573.pdf) and ATCC 1656 broth (http://www.atcc.org/mediapdfs/1656.pdf), respectively, according to the manufacturer’s instructions. Both strains were grown in broth for 18-36 h in order to obtain a cell concentration of $10^6$-$10^7$ colony forming unit (CFU) ml$^{-1}$. *Alicyclobacillus*
*acidoterrestris* ATTC 49025 was plated on acidified Potato Dextrose Agar (Becton Dickinson and Company, Sparks, MD) and incubated at 48°C for 48 h for plate counting. The following strains used in the specificity assay were grown for 24 h in media and growth conditions as specified: *Escherichia coli* DH-5α (Invitrogen, Carlsbad, CA) and INVαF’ (Invitrogen, Carlsbad, CA) in Miller LB broth (Fisher Chemicals, Fairlawn, NJ), 37°C; *Lactococcus lactis* subsp. *lactis* 2301 (Walsh and McKay, 1981) in M17-G (Becton Dickinson and Company, Sparks, MD), 30°C; *Pseudomonas putida* ATCC 49451 (ATCC, Manassas, VA) in Tryptic Soy Broth (TSB, Becton Dickinson and Company, Sparks, MD), 30°C; *Listeria monocytogenes* V7 (Yousef et al., 1988) in TSB, 37°C; *Geobacillus stearothermophilus* ATCC 10149 (ATCC, Manassas, VA) in Difco Nutrient Broth (Becton Dickinson and Company, Sparks, MD), 55°C; *Bacillus subtilis* OSU494 (Khadre et al., 2001) in Difco Nutrient broth, 37°C. Frozen stocks of all strains were stored in their respective media, supplemented with 20% glycerol and kept at -80°C. Cultures were kept at 4°C and maintained by biweekly transfers in corresponding media.

**4.3.2 DNA extraction, gene cloning and DNA sequencing**

For DNA extraction, cells were collected from 1 ml of bacterial culture or its dilutions by micro-centrifugation at 5400 ×g for 10 min. Collected cells were treated with 20 mg ml⁻¹ of lysozyme (Sigma Chemical CO., St Louis, MO) in enzymatic lysis buffer (20 mmol l⁻¹ Tris.Cl, pH 8.0, 2 mmol l⁻¹ EDTA, and 1.2% Triton®X-100) for 45 min at 37°C. Genomic DNA was extracted using a commercial isolation kit (DNeasy® Tissue
Kit, Qiagen, Valencia, CA) and eluted with 100 µl of elution buffer following the instructions from the manufacturer.

An established approach (Wang et al., 2000) to clone microbial genes with defined functions but unknown sequences was followed to clone a fragment of the squalene-hopene cyclase (SHC) encoding gene, \(\text{she}\) (Wendt et al., 1997; Kannenberg and Poralla, 1999), from representative strains of \textit{Alicyclobacillus} spp. Basically, SHC protein sequences from several microorganisms including \textit{A. acidocaldarius} ATCC27009 (GenBank accession number AAA75452), \textit{A. acidoterrestris} DSM3922 (CAA61950), \textit{B. subtilis} 168 (CAB13824), \textit{Dictyostelium discoideum} taxon:44689 (cycloartenol synthase, AAF80384), \textit{Synechocystis} sp. PCC6803 (BAA17978), and \textit{Streptomyces coelicolor} A3(2) (CAB39697) were aligned, and conserved amino acid sequences were identified. Two degenerate primers HLSHC1 (5’ GGNGGNTGGATGTTYCARGC 3’) and HLSHC2 (5’ YTCNCCCCANCCNCCRTC 3’) (Y=C+T; R=A+G; N=A+T+C+G) were derived based on the conserved amino acid sequences GGWMFQA (only conserved for \textit{Alicyclobacillus}) and DGGWGE (conserved for all the strains except \textit{Bacillus}) within the SHC proteins. The \textit{she} fragments were amplified by conventional PCR using this set of primers and the genomic DNA from \textit{A. acidocaldarius} ATCC 43030 and \textit{A. acidoterrestris} ATCC 49025 as templates. PCR conditions were: one cycle at 95°C for three min, followed by 30 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for one min, with a final extension at 72°C for seven min using a thermal cycler (iCycler™, Bio-Rad, Hercules, CA). PCR products were purified using a commercial purification kit (QIAquick®, Qiagen, Valencia, CA) following manufacturer’s instruction. Purified PCR products were cloned into pCR 2.1 vectors and transformed into \textit{E. coli} INV\(\alpha\)F’
competent cells using a cloning kit (TA Cloning®, Invitrogen, Carlsbad, CA). Recombinant plasmids were recovered using a miniprep kit (QIAprep®, Qiagen, Valencia, CA). DNA sequences were determined using a DNA analyzer (ABI PRISM® 3700, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio State University.

4.3.3 DNA sequence analysis

A commercial program (MegAlign, DNASTAR, Inc., Madison, WI) was used in DNA and protein sequence alignment. Comparison of primers and probes prepared in this study, with sequences from the GenBank database, was done using a sequence comparison algorithm (Altschul et al., 1990).

4.3.4 Real-time PCR conditions

For real-time PCR, the reaction was conducted in thin-wall microcentrifuge tubes. The reaction mixture contained 25 µl of the premixed commercial PCR reagent iQ™ Supermix (100mM KCl, 40mM Tris-HCl, pH8.4, 1.6mM dNTPs, iTaq DNA polymerase, 50 units/ml, 6mM MgCl₂ and stabilizers, Bio-Rad, Hercules, CA), 0.025 nmol of each primer, 0.03 nmol probe, 10 µl genomic DNA extract and ddH₂O in a final volume of 50 µl. PCR was performed one cycle at 95°C for three min followed by 33-40 cycles of 95°C for 30 s, 55°C for one min, using the Real-Time PCR system (iCycler, Bio-Rad, Hercules, CA).
4.3.5 Specificity and sensitivity analyses

Specificity of the developed detection system was tested using spoilage
Alicyclobacillus spp. and selected foodborne microorganisms. Alicyclobacillus
acidocaldarius and A. acidoterrestris were grown in ATCC573 broth or ATCC1656
broth at 48°C for 36 h with a final population around of 10^7 CFU ml⁻¹, approximately.
Additionally, B. subtilis OSU 494, E. coli DH5α, L. lactis subsp. lactis 2301, G.
stearothermophilus ATCC 10149 and P. putida ATCC49451, were grown under
the conditions described earlier. Genomic DNA was extracted from these cultures using the
previously discussed DNA extraction protocol. One-tenth of the DNA eluate was used as
a template and the real-time PCR amplification was carried out based on conditions
described earlier.

The sensitivity of the real-time PCR assay was determined using A.
acidoterrestris populations of different cell densities. The bacterium was inoculated (2%)
in ATCC1656 broth, and the culture was incubated at 48°C for 18 h; final absorbance
(O.D.₆₀₀) was approximately 0.34, which corresponds to a population of 10^6 CFU ml⁻¹,
approximately. Cells from 1ml culture were collected by centrifugation at 5400×g for 10
min, and resuspended in one ml 0.85% saline solution or shelf-stable reconstituted apple
juice (MinuteMaid®, Coca-cola Inc., Atlanta, GA). These “original” cell suspensions
were further serially diluted in saline and apple juice, respectively. One milliliter of each
dilution was centrifuged and the DNA of collected cells was extracted as indicated earlier.
One-tenth (10 µl) of the eluted DNA was used as a template and the real-time PCR
amplification was carried out as described earlier. To compare the real-time PCR output
with results of conventional plate counting approach, 100 µl from the 10⁻³ to 10⁻⁵
dilutions of the original cell suspension were plated on acidified Potato Dextrose Agar (Becton, Dickinson and Company, Sparks, MD). Plates were incubated at 48°C for 48 h followed by colony counting.

4.4 Results

4.4.1 The primer-and-probe set used in the real-time PCR assay

This study targeted a gene (shc) encoding squalene-hopene cyclase (SHC), which is a key enzyme in hopanoid biosynthesis, for real-time PCR primer-and-probe set development to detect spoilage alicyclobacilli. Hopanoids are important membrane components of *Alicyclobacillus* spp. involved in maintaining membrane fluidity and stability and therefore is critical for the biological functionality of the organisms at extreme environmental conditions (Kannenberg and Poralla, 1999).

Because information about *Alicyclobacillus* SHC gene sequence in the DNA database is limited and insufficient for the primer-and-probe development, we have cloned an *shc* gene fragment from two additional *Alicyclobacillus* spp. strains. Using the degenerate primers HLSHC1 and HLSHC2, and the genomic DNA from *A. acidocaldarius* ATCC 43030 or *A. acidoterrestris* ATCC 49025 as the template, an *shc* fragment (708 bp) was amplified by conventional PCR from each strain. The *shc* fragments from both strains were cloned and their DNA sequences were determined as described in methodology, and sequence data were deposited in the GenBank (Accession numbers AY563514 and AY563515, for *A. acidocaldarius* ATCC 43030 and *A. acidoterrestris* ATCC 49025, respectively).
In order to develop a primer-and-probe set within the \textit{shc} region suitable for the real-time PCR detection, DNA sequences from selected strains, including the two from this study, were aligned using a commercial program (MegAlign, DNASTAR, Inc., Madison, WI). Three conserved oligonucleotides were derived including the forward primer 5' ATGCAGAGYTCGAACG 3' and the reverse primer 5' AAGCTGCCGAARCACTC 3' flanking an amplicon of 149 bp, and the probe 5'TCRGARGACGTCACCGC3' (Fig. 4.1). The reporter dye, FAM (6-carboxyfluorescein), was conjugated at the 5' end of the probe. The quencher dye, Black Hole Quencher (BHQ) dye I, was conjugated at the 3' end. The BHQ dye had broader quenching spectrum and a lower signal-to-noise ratio than that of other quenching dyes and therefore was selected (Ibekwe \textit{et al.}, 2002). The primers were synthesized by Sigma-Genosys (Sigma-Genosys, The Woodlands, TX). The FAM – and-BHQ labeled probe was synthesized by Biosearch Technologies (Biosearch technologies, Novato, CA) and used in the real-time PCR assay.

\textbf{4.4.2 Specific detection of spoilage \textit{Alicyclobacillus} spp.}

Real-time PCR assays were performed to determine the specificity of the \textit{shc}-targeting primers-and-probe for \textit{A. acidocaldarius} and \textit{A. acidoterrestris}. Representative strains of selected bacteria commonly found associated with foods were included in the study to test the possibility of cross-reactions by the primer-and-probe set. Assays were performed in triplicate, and a representative real-time PCR chart is shown in Fig.4.2. According to the output charts, positive curves were identified in samples containing \textit{A. acidocaldarius} ATCC 43030 and \textit{A. acidoterrestris} ATCC 49025. Common foodborne
microorganisms including *G. stearothermophilus*, a bacterium closely related to *Alicyclobacillus* spp., did not cause cross reactivity. Although only a few representative strains were used in the laboratory specificity studies, further computer-based specificity search was conducted covering all the deposited DNA sequences available through the National Center for Biotechnology Information (NCBI). No combination of the above three oligonucleotides (104 hits for the forward primer with 82% or higher identity; 95 hits for the reverse primer with 83% or higher identity; 161 hits with 77% or higher identity for the probe) was found in any microorganisms but *A. acidocaldarius* and *A. acidoterrestris*. These results suggest that under proper stringent conditions, the combination of the sequences of the oligonucleotide primers and probe used in the study are distinctive enough to detect *A. acidocaldarius* and *A. acidoterrestris* strains but not other microorganisms.

### 4.4.3 Levels of detection in apple juice and saline

Experiments were conducted to determine the level of detection, i.e., sensitivity testing, for targeted bacteria using the newly developed real-time PCR method. All experiments were repeated for at least three times and a representative detection chart was presented as Fig. 4.3. Apple juices containing $4.0 \times 10^6$ to $4.0 \times 10^1$ CFU ml$^{-1}$ of *A. acidoterrestris*, corresponding to $4.0 \times 10^5$ to $4.0 \times 10^0$ CFU per PCR reaction, tested positive by the real-time PCR method (Fig. 4.3). Time elapsed before appearance of a positive signal varied with the cell density in the juice; a greater number of thermal cycles were needed to produce detectable amplicons from samples containing low populations than from those with high cell density. From all repeats, less than $1.0 \times 10^1$
CFU $A. \text{acidoterrestris}$ per reaction tested positive by the real-time PCR method. Similar levels of detection were obtained when cells of $A. \text{acidoterrestris}$ were suspended in saline instead of apple juice (chart not shown).

4.5 Discussion and Conclusion

Rapid, specific and sensitive detection of microorganisms has always been a challenge to the food industry and real-time PCR technology has the potential to address this issue. In this study, a set of primer-and-probe, targeting the $shc$ gene, was developed and tested using $A. \text{acidocaldarius}$ and $A. \text{acidoterrestris}$. This group of bacteria is involved in the spoilage of fruit juices; therefore, the study is of industrial significance. Using the newly developed method, the presence of $< 1.0 \times 10^1$ CFU per reaction can be readily detected without pre-extraction or enrichment procedures. This result is comparable to data from previous studies reporting real-time PCR detection limits from $< 1.0 \times 10^2$ to $< 1.0 \times 10^1$ CFU per reaction (Bassler et al., 1995; Nogva et al., 2000; Hein et al., 2001). This detection limit is also comparable with results obtained by the conventional culturing methods. In the current study, similar levels of detection were observed when $A. \text{acidoterrestris}$ were suspended in saline and apple juice, which indicated that ingredients from apple juice did not cause significant inhibition to the real-time PCR reaction using the described procedures. As illustrated in Fig. 3, the new method detected $< 1.0 \times 10^1$ CFU $A. \text{acidoterrestris}$ per reaction. However, this result should be cautiously interpreted. Counts of $A. \text{acidoterrestris}$ were done using PDA medium and not all viable cells might have been recovered. Additionally, DNA from dead cells may interfere with the real-time PCR detection procedure.
*Alicyclobacillus* spp. may be present in juice products as spores or vegetative cells. This study illustrates the feasibility of using real-time PCR to detect vegetative cells of *Alicyclobacillus* spp. To detect the spore forms, additional steps to induce spore germination and outgrowth should be included before the DNA is extracted. Although these modifications add time to the detection procedure, the real-time PCR approach remains less time consuming than the conventional methods.

For potential industrial implementation, the detection limit (and the procedure, in general) can possibly be improved further by increasing sample size and concentrating the microbial cells through various means. The proposed improvement is in fact a preferred practice since the industrial sample size usually varies from 25 ml to one liter. Based on our experience, however, inhibition to PCR reaction occurs when a concentration procedure is included without additional sample treatment before DNA extraction. This was further confirmed by the observation that a rinsing or a dilution step is needed for proper detection of *Alicyclobacillus* spp. in an industrial apple juice concentrate, the raw material for juice processing (data not shown). A set of membrane filtration units, enabling large food particles separation, microbial cell concentrating and rinsing off inhibitory factors could be usefully coupled with the real-time Taqman® system for industrial microbial detection applications. Further studies in our laboratory suggested that the real-time PCR based detection systems also functioned properly in orange juice and other juice products (data not shown).

In conclusion, we demonstrated the great potential of real-time Taqman PCR-based method to achieve rapid and specific detection of spoilage microorganisms in apple juice, with the detection sensitivity level at least comparable to conventional methods.
Using the above she-based real-time PCR method, detection of $< 1.0 \times 10^1$

_Alicyclobacillus_ CFU in defined volume of juice sample can be achieved within 3-5 h as oppose to 48 h or longer by conventional methods. Such a system will be very useful for industrial application from raw material screening to final product quality control. The technology being developed by the Ohio State University is patent pending.
4.6 References


Figure 4.1 Alignment of *shc* fragments from *Alicyclobacillus acidocaldarius* ATCC43030 (*A. ac 43030*), *A. acidocaldarius* ATCC27009 (*A. ac 27009*), *A. acidoterrestris* ATCC49025 (*A. at 49025*), *A. acidoterrestris* DSM3922 (*A. at DSM3922*). Boxed nucleotide sequences were used to design PCR primers and probe.
Figure 4.2 Real-time PCR amplification of *she*-specific product using the developed primer-and-probe set in testing common foodborne bacteria. ▲ *Alicyclobacillus acidocaldarius* ATCC 43030; ■ *A. acidoterrestris* ATCC49025; Bacteria that gave no response (horizontal lines) are *Bacillus subtilis* OSU 494, *Pseudomonas putida* ATCC49451, *Escherichia coli* DH5α, *Listeria monocytogenes* V7, *Lactococcus lactis* 2301 and *Geobacillus stearothermophilus* ATCC 10149. CF RFU: curve fit relative fluorescence units.
Figure 4.3 Detection of real-time PCR amplicons obtained using different inoculation of Alicyclobacillus acidoterrestris ATCC49025 in apple juice. Symbols: ■ 4.0×10^5 CFU per reaction; □ 4.0×10^4 CFU per reaction; ♦ 4.0×10^3 CFU per reaction; ● 4.0×10^2 CFU per reaction; ○ 4.0×10^1 CFU per reaction; ▼ uninoculated juice (blank control). CF RFU: curve fit relative fluorescence units.
CHAPTER 5

DEVELOPING PRIMER-AND-PROBE SETS FOR TAQMAN REAL-TIME PCR-BASED RAPID DETECTION OF *LISTERIA MONOCYTOGENES*

5.1 Abstract

*Listeria monocytogenes* is a common foodborne pathogen found in various foods, and can cause listeriosis particularly in high-risk population. To minimize the risk of listeriosis, proper monitoring system is essential for early detection of potential problems.

The aim of this project was to develop primer-and-probe sets for a real-time PCR-based monitoring system of *L. monocytogenes* in meat products.

Two genes on the virulence gene cluster, listeriolysin O-encoding gene *hly* and the positive regulatory factor-encoding gene *prfA* were targeted for specific primer-and-probe development, respectively. Gene fragments from representative strains were cloned, and PCR primers and probe were designed by DNA sequence comparison. Selected bacteria were examined for cross-reactivity by the new method.

Using real-time PCR, the presence of representative *L. monocytogenes* strains were detected by the newly developed Taqman® real-time PCR-based method without cross reactivity with other common foodborne microorganisms. These primer-and-probe sets can further be used to investigate *L. monocytogenes* virulence gene expression in various environments using real-time PCR.
5.2 Introduction

*L. monocytogenes* is a foodborne pathogen causing listeriosis mainly in high-risk groups, including pregnant women, neonates, and immunocompromised adults (Slutsker and Schuchat 1999). The outbreaks of human listeriosis were reported in many countries since 1980s and involved various kinds of foods, including milk, cheese, vegetables, coleslaw, seafood, raw meat and ready-to-eat meat (Ryser 1999). Listeriosis symptoms are variable and depend on the individual's susceptibility. Symptoms may be limited to fever, fatigue, nausea, vomiting and diarrhea; however, these symptoms can precede a more serious illness: the more serious forms of listeriosis can result in meningitis (brain infections) and septicemia (bacteria in the bloodstream). Pregnant women may contract flu-like symptoms of listeriosis; complications can result in miscarriage, stillbirth, or septicemia or meningitis in the newborn (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_g.htm#symptoms). Every year there are about 5000 cases of listeriosis, which has a mortality rate of approximately 20% to 30% (Gellen et al., 1999).

The high-case fatality rate and increasing outbreaks resulted in increased attention to *L. monocytogenes*. It is a gram-positive rod and was reported to have an optimal growth temperature between 30 and 37°C and a highest growth temperature at 45°C, optimal aw about 0.97, optimal pH from neutral to slightly alkaline (Lou and Yousef 1999). However, *L. monocytogenes* is a psychrotrophic bacterium and resists to heat, salt, nitrite and acidity much better than many organisms. The lowest growth temperature of *L. monocytogenes* was reported to be close to 0°C (Hudson et al., 1994) in an appropriate medium. It was also reported to be an acid-tolerant bacterium and can grow in water.
activity as low as 0.9 (Reimer et al., 1988). The above characteristics indicate *L. monocytogenes* can survive and even multiply in various food products even in refrigeration temperature, which makes it very dangerous in ready-to-eat foods.

Due to the potential risk associated with this bacterium, neither the Food Safety and Inspection Service (FSIS) nor FDA will accept any detectable *L. monocytogenes* in cooked, ready-to-eat food ([http://vm.cfsan.fda.gov/~mow/fsislist.html](http://vm.cfsan.fda.gov/~mow/fsislist.html)). This is called "zero tolerance" for the *L. monocytogenes*. Therefore, a sensitive and specific detection method is highly in needed. Currently, the standard FDA and USDA methods use selective medium to enrich and isolate *L. monocytogenes* from food sample, and the suspicious bacterial colonies are further confirmed by biochemical tests. Such methods have improved specificity and sensitivity for the detection of *L. monocytogenes* from various foods. However, these methods are still very time-consuming, the whole procedure takes approximately one week.

In order to fulfill the requirement of a rapid, sensitive and specific detection method of *L. monocytogenes* from food samples, the efforts of applying novel techniques including ELISA, nucleic-acid probe hybridization and regular PCR have never been stopped. However, the sensitivity of ELISA and nucleic-acid probe hybridization is still low, the regular PCR obtained great sensitivity but the specificity is not ideal because of non-stringent amplification.

Recently advancement in real-time PCR provides a better technique platform for food scientists to develop efficient detection method for microorganisms. Among Sybr-Green, Molecular beacon, FRET and Taqman real-time PCR, Taqman has the advantages of high specificity and medium cost, and has been adapted for various applications from
clinical diagnostics to environmental microbial survey (Cockerill 2002; Hanna et al., 2005). Basically it targets signature genes of microorganisms and the amplification of the signature gene reflects the presence of the microorganisms.

*L. monocytogenes* has its unique virulence genes that are good candidates for developing primer-and-probe sets used in Taqman real-time PCR. Several virulence genes, including *hly*, *actA*, *plc*, and *prfA*, are located on a virulence gene cluster (Engelbrecht et al., 1996; Kuhn and Goebel 1999). It has been reported that the invasion of *L. monocytogenes* to different cells types may be related to *inlA*, *inlB*, *inlc*, *actA* genes (Gaillard et al., 1991; Dramsi et al., 1995; Engelbrecht et al., 1996; Alvarez-Dominguez et al., 1997; Kuhn and Goebel 1999). However the exact functions of these gene-encoding proteins are yet to be revealed. The *hly*-encoding virulence factor listeriolysin O, is a secreted protein of 58-60KD belonging to a family of pore-forming sulphhydryl cytolysin causing hemolysis. A clear correlation between hemolysis and virulence has been established. All virulent strains have been reported to be hemolytic and non-hemolytic strains are avirulent (Gaillard et al., 1986 and Katharious et al., 1987). As a regulatory factor of several virulence genes, the *prfA* encoding protein plays an important role in the virulence of *L. monocytogenes*.

The objective for this study was to develop the methodology for detection of *L. monocytogenes* in meat products using a Taqman®-based real-time PCR approach. This involves identifying two target genes in representative *L. monocytogenes* strains and developing two primer-and-probe sets suitable for the real-time PCR system. The study also examines specificity of the primer-and-probe sets to the targeted bacteria.
5.3 Materials and methods

5.3.1 Bacterial strains and growth conditions

*L. monocytogenes* V7 (serotype 4b, clinical isolate, Yousef *et al.*, 1983) and ScottA (serotype 1/2a, milk isolate) were grown in Tryptic Soy Broth (TSB, Becton Dickinson and Company, Sparks, MD) for 18-36 h in order to obtain a cell concentration of $10^9$-to $10^{10}$ colony forming unit (CFU) ml$^{-1}$. The following strains used in the specificity assay were grown for 24 h in media and growth conditions as specified: *Escherichia coli* DH-5α (Invitrogen, Carlsbad, CA) in Miller LB broth (Fisher Chemicals, Fairlawn, NJ), 37°C; *Lactococcus lactis* subsp. *lactis* 2301 (Walsh and McKay, 1981) in M17-G (Becton Dickinson and Company, Sparks, MD), 30°C; *Pseudomonas putida* ATCC 49451 (ATCC, Manassas, VA) in TSB (Becton Dickinson and Company, Sparks, MD), 30°C; *Listeria innocua*, *L. monocytogenes* 8517, 8578, 8576 and 8732 (meat isolates), F2365 (serotype 4b, cheese isolate, Mascola *et al.*, 1988) in TSB (Becton Dickinson and Company, Sparks, MD), 37°C. Frozen stocks of all strains were stored in their respective media, supplemented with 20% glycerol and kept at -80°C. Cultures were kept at 4°C and maintained by biweekly transfers in corresponding media.

5.3.2 DNA extraction, gene cloning and DNA sequencing

For DNA extraction, cells were collected from 1 ml of bacterial culture or its dilutions by micro-centrifugation at 5400 ×g for 10 min. Collected cells were treated with 20 mg ml$^{-1}$ of lysozyme (Sigma Chemical CO., St Louis, MO) in enzymatic lysis buffer (20 mmol l$^{-1}$ Tris.Cl, pH 8.0, 2 mmol l$^{-1}$ EDTA, and 1.2% Triton $^o$X-100) for 45 min at
37°C. Genomic DNA was extracted using a commercial isolation kit (DNeasy® Tissue Kit, Qiagen, Valencia, CA) and eluted with 100 µl of elution buffer following the instructions from the manufacturer.

Two primers pairs Hly-U (5’ GCACCACCAGCTCCGC3’) and Hly-D (5’CCACTAATGTTACTGCGT3’); PrfA-U (5’ CCAATGGGATCCAC AAG3’) and PrfA-D (5’CCCCAAGTAGCAGGACATGC3’) were derived based on the alignments of published DNA sequences of hly gene and prfA gene from L. monocytogenes and other Gram-positive bacteria. Gene fragments from the same region of representative strains, L. monocytogenes V7 (serotype 4b, clinical isolate) and Scott A (serotype 1/2a, milk isolate) were amplified by PCR using the primer pairs. The hly fragments and prfA fragments were amplified by conventional PCR using this set of primers and the genomic DNA from L. monocytogenes V7 and Scott A as templates. PCR conditions were: one cycle at 95°C for three min, followed by 30 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for one min, with a final extension at 72°C for seven min using a thermal cycler (iCycler™, Bio-Rad, Hercules, CA). PCR products were purified using a commercial purification kit (QIAquick®, Qiagen, Valencia, CA) following manufacturer’s instruction. Purified PCR products were cloned into pCR 2.1 vectors and transformed into E. coli INVαF’ competent cells using a cloning kit (TA Cloning®, Invitrogen, Carlsbad, CA). Recombinant plasmids were recovered using a miniprep kit (QIAprep®, Qiagen, Valencia, CA). DNA sequences were determined using a DNA analyzer (ABI PRISM® 3700, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio State University.
5.3.3 DNA sequence analysis

A commercial program (MegAlign, DNASTAR, Inc., Madison, WI) was used in DNA and protein sequence alignment. Comparison of primers and probes prepared in this study, with sequences from the GenBank database, was done using a sequence comparison algorithm (Altschul et al., 1990).

5.3.4 Real-time PCR conditions

For real-time PCR, the reaction was conducted in thin-wall microcentrifuge tubes. The reaction mixture contained 25 µl of the premixed commercial PCR reagent iQ™ Supermix (100mM KCl, 40mM Tris-HCl, pH8.4, 1.6mM dNTPs, iTaq DNA polymerase, 50 units/ml, 6mM MgCl₂ and stabilizers, Bio-Rad, Hercules, CA), 0.018 nmol of each primer, 0.015 nmol probe, 5 µl genomic DNA extract and ddH₂O in a final volume of 50 µl. PCR was performed one cycle at 95°C for three min followed by 30 cycles of 95°C for 30 s, 55°C or 57°C for 45 seconds, using the Real-Time PCR system (iCycler, Bio-Rad, Hercules, CA).

5.3.5 Specificity analyses

Specificity of the developed detection system was tested using *L. monocytogenes* strains and selected foodborne microorganisms. All of the microorganisms were grown under the conditions described earlier. Genomic DNA was extracted from these cultures using the previously discussed DNA extraction protocol. One-tenth of the DNA eluate was used as a template and the real-time PCR amplification was carried out based on conditions described earlier. Representative strains of selected bacteria commonly found
associated with foods were included in the study to test the possibility of cross-reactions by the primer-and-probe set. Assays were performed in triplicate.

5.4 Results

5.4.1 Developing the primer-and-probe set used in the real-time PCR assay

This study targeted a gene hly encoding listeriolysin O, which is a secreted protein causing hemolysis, and the gene prfA encoding a virulence regulatory factor, for real-time PCR primer-and-probe set development to detect L. monocytogenes. Because L. monocytogenes hly and prfA gene sequences were limited in the DNA database, we cloned the hly and prfA gene fragments from two additional L. monocytogenes strains. Based on published DNA sequences from L. monocytogenes and other Gram-positive bacteria, DNA oligonucleotides were derived based on homology and used as primers to amplify fragments from the target hemolysin gene hly and positive regulator gene prfA from both strains of L. monocytogenes. The amplified hly fragments were about 440 bp and the cloned prfA fragments were about 600 bp (Fig.5.1 and Fig.5.2).

In order to develop primer-and-probe sets within the hly region suitable for the real-time PCR detection, DNA sequences from selected strains, including the two from this study, were aligned using a commercial program (MegAlign, DNASTAR, Inc., Madison, WI). Three conserved oligonucleotides were derived including the forward primer (5’AGTCCTAAGACGCAATC3’), and the reverse primer (5’TAACCTTTTCTTGCGGC3’), flanking an amplicon of 134 bp, and the probe 5’CCA CGG AGA TGC AGT GAC 3’ (Fig.5.3). The reporter dye, FAM (6-carboxyfluorescein), was conjugated at the 5’ end of the probe. The quencher dye, Black
Hole Quencher (BHQ) dye I, was conjugated at the 3’ end. The BHQ dye had broader quenching spectrum and a lower signal-to-noise ratio than that of other quenching dyes and therefore was selected (Ibekwe et al., 2002).

In a same fashion, three prfA specific oligonucleotides, namely the forward primer (5'AAACTCCTGATGGCATC3'), the reverse primer (5'CACGATAACTTTCTCTTGC 3'), flanking an amplicon of 131 bp, and the probe 5'TCG CAC ATA GCT CAG CTG3' (Fig.5.4), were derived. The reporter dye, CY5, was conjugated at the 5’ end of the probe. The quencher dye, Black Hole Quencher (BHQ) dye II, was conjugated at the 3’ end. The primers were synthesized by Sigma-Genosys (Sigma-Genosys, The Woodlands, TX). The FAM-and-BHQ I, and CY5-and-BHQII labeled probes were synthesized by Biosearch Technologies (Biosearch technologies, Novato, CA) and used in the real-time PCR assay.

5.4.2 Specific detection of L. monocytogenes

Real-time PCR assays were performed to determine the specificity of the hly targeting primer-and-probe for L. monocytogenes. Positive curves were identified in samples containing L. monocytogenes V7, ScottA, F2365, meat isolates 8517, 8576, 8578 and 8732. Common foodborne microorganisms including L. innocua, a bacterium closely related to L. monocytogenes, did not cause cross reactivity (Data not shown). Although only a few representative strains were used in the laboratory specificity studies, further computer-based specificity search was conducted covering all the deposited DNA sequences available through the National Center for Biotechnology Information (NCBI). No combination of the above three oligonucleotides (250 hits for the forward primer with
73% or higher identity; 250 hits for the reverse primer with 78% or higher identity; 250 hits with 78% or higher identity for the probe) was found in any microorganisms but \textit{L. monocytogenes}.

Real-time PCR assays were also performed to determine the specificity of the \textit{prfA} targeting primer-and-probe for \textit{L. monocytogenes}. A representative real-time PCR chart is shown in Fig. 5.5. According to the output charts, positive curves were identified in samples containing \textit{L. monocytogenes}V7, ScottA, F2365, meat isolates 8517, 8576, 8578 and 8732. Common foodborne microorganisms including \textit{L. innocua}, a bacterium closely related to \textit{L. monocytogenes}, did not cause cross reactivity. Although only a few representative strains were used in the laboratory specificity studies, further computer-based specificity search was conducted covering all the deposited DNA sequences available through the National Center for Biotechnology Information (NCBI). No combination of the above three oligonucleotides in \textit{prfA} region was found in any microorganisms but \textit{L. monocytogenes}.

These results suggest that under proper stringent conditions, the combination of the sequences of the oligonucleotide primers and probes used in the study are distinctive enough to detect \textit{L. monocytogenes} strains but not other microorganisms.

\textbf{5.5 Discussion and Conclusion}

Rapid, specific and sensitive detection of microorganisms has always been a challenge to the food industry and real-time PCR technology has the potential to address this issue. In this study, two sets of primer-and-probe, targeting the \textit{hly} gene and \textit{prfA}
gene, were developed for the rapid detection of *L. monocytogenes* using real-time PCR assay. Using the primer and probe sets, samples with representative pathogenic strains V7, Scott A, and F2365 were identified as positive while samples containing other foodborne bacteria including *L. innocua*, *L. lactis*, *P. putida* and *E. coli* were found to be negative.

Four *L. monocytogenes* isolates isolated by the Ohio Department of Agriculture from meat samples were also examined. Using the *hly* and *prfA* specific primer and probe set, four of them were all tested positive.

Several foodborne bacteria including *L. innocua* have been tested using the two sets of primer-and-probe. The sequences of the *hly* and *prfA* fragment for primers and probes development were also compared with the sequences from other *Listeria* species available in the GenBank. Fig. 5.3 showed that the primers and probe developed to target *L. monocytogenes hly* fragment are conservative in all the virulent strains of *L. monocytogenes*, but have at least 5 out of 18 bp difference from the non-pathogenic *L. seeligeri* ATCC 35967 (GenBank accession number AY 878348) per oligo fragment. Fig.5.4 showed that the primers and probe developed to target *prfA* are only conserved intra-species, but different from *L. ivanovii* (Ramage 1999).

Sensitivity analysis has also been conducted in our laboratory using the developed primer-and-probe sets for *L. monocytogenes* detection. The applicability of the *L. monocytogenes* detection system for food applications was further validated in roast beef. The availability of the two sets of primer-and-probe sets enables future detection approaches using multiplex reactions. These primer-and-probe sets can also be used to study virulence gene expression using quantitative real-time PCR.
5.6 References


Figure 5.1 Agarose gel (0.9%) electrophoresis of amplified *L. monocytogenes* hly fragments using standard PCR. Lane 1, 1kb plus DNA ladder; Lane 2, *hly* amplification product from *L. monocytogenes* V7; Lane 3, *hly* amplification product from *L. monocytogenes* ScottA.
Figure 5.2. Agarose gel (0.9%) electrophoresis of prfA fragments amplified from *L. monocytogenes* using standard PCR. Lane 1, 1kb plus DNA ladder; Lane 2, prfA amplification product from *L. monocytogenes* V7; Lane 3, prfA amplification product from *L. monocytogenes* ScottA.
AGTCCTAAGACGCCAATCAGAAAAGAAACACCGGGATGAATTCGAT 1 V7
AGTCCTAAGACGCCAATCAGAAAAGAAACACCGGGATGAATTCGAT 2 ScottA
AGTCCTAAGACGCCAATCAGAAAAGAAACACCGGGATGAATTCGAT 3 USDA F-2365
AGTCCTAAGACGCCAATCAGAAAAGAAACACCGGGATGAATTCGAT 4 ATCC15313
AGTCCTAAGACGCCAATCAGAAAAGAAACACCGGGATGAATTCGAT 5 EGD-4
ACACCAACACAGGTCTAGAAAAAGCATCGGGAAAGATTAAT 6 L. seeligeri

AAGTATATACAAGGATTGGATTACAATAAAAACAATGTATTAGTA 1
AAGTATATACAAGGATTGGATTACAATAAAAACAATGTATTAGTA 2
AAGTATATACAAGGATTGGATTACAATAAAAACAATGTATTAGTA 3
AAGTATATACAAGGATTGGATTACAATAAAAACAATGTATTAGTA 4
AAGTATATACAAGGATTGGATTACAATAAAAACAATGTATTAGTA 5
AAATATATTTGGGGATTAAACTATGATAAAAATAGTATTCTGTC 6

TACCACGGAGATGCGAGAACATTGTCGCCGGAAGAAAGCTTA 1
TACCACGGAGATGCGAGAACATTGTCGCCGGAAGAAAGCTTA 2
TACCACGGAGATGCGAGAACATTGTCGCCGGAAGAAAGCTTA 3
TACCACGGAGATGCGAGAACATTGTCGCCGGAAGAAAGCTTA 4
TACCACGGAGATGCGAGAACATTGTCGCCGGAAGAAAGCTTA 5
TACCACGGAGATGCGAGAACATTGTCGCCGGAAGAAAGCTTA 6

Figure 5.3. Alignment\(^a\) of 134 bp priming region flanked by hly-FP (5’AGTCCTAAGACGCCAATC3’), hly Probe (5’ CCACGGAGATGCAGTGAC 3’), and hly-RP (5’ TAACCGAGGAATGAC 3’).\(^b\) Boxed nucleotide sequences were used to design PCR primers and probe; The nucleotides varied from the designed primers and probe were underlined.

\(^a\) Sequences in the alignment are \(hly\) sequences from the following organisms (GenBank accession numbers follow if applicable): 3)NC-002973, 4)AY750900, 5)NC-003210, 6)AY878348. \(^b\) Note hlyA-RP is in 5’ to 3’ orientation in alignment. Actual primer sequence is the reverse complement.
AAACTCCTGATGGCATCAAGATTACACTGGATAATTTAACAATGC 1 V7
AAACTCCTGATGGCATCAAGATTACACTGGATAATTTAACAATGC 2 ScottA
AAACTCCTGATGGCATCAAGATTACACTGGATAATTTAACAATGC 3 HCC23
AAACTCCTGATGGCATCAAGATTACACTGGATAATTTAACAATGC 4 Atcc15313
AAACTCCTGATGGCATCAAGATTACACTGGATAATTTAACAATGC 5 EGD-4
AAACTCCTGATGGCATCAAGATTACACTGGATAATTTAACAATGC 6 \textit{L.ivanovii} NRRL 33017
AAACTCCTGATGGCATCAAGATTACACTGGATAATTTAACAATGC 7 \textit{L.ivanovii} NRRL 33021

AGGAGTTAGGATATTTCAAGGGCCTTAGCATAGTCAGCTGTTA 1
AGGAGTTAGGATATTTCAAGGGCCTTAGCATAGTCAGCTGTTA 2
AGGAGTTAGGATATTTCAAGGGCCTTAGCATAGTCAGCTGTTA 3
AGGAGTTAGGATATTTCAAGGGCCTTAGCATAGTCAGCTGTTA 4
AGGAGTTAGGATATTTCAAGGGCCTTAGCATAGTCAGCTGTTA 5
AGGAGTTAGGATATTTCAAGGGCCTTAGCATAGTCAGCTGTTA 6
AGGAGTTAGGATATTTCAAGGGCCTTAGCATAGTCAGCTGTTA 7

Figure 5.4. Alignment\textsuperscript{a} of 131 bp priming region flanked by prf-FP (5’AAACTCCTGATGGCATC3’), prf Probe (5’TCGCACATAGTCAGCTG 3’), and prf-RP (5’CAGATACACTCGCTTGC 3’).\textsuperscript{b} Boxed nucleotide sequences were used to design PCR primers and probe; The nucleotides varied from the designed primers and probe were underlined.

\textsuperscript{a} Sequences in the alignment are prf sequences from the following organisms (GenBank accession numbers follow if applicable): 3) AY878649, 4) AY750900, 5) NC-003210, 6) AY510072, 7) AY510073 (Ward \textit{et al.}, 2004) . \textsuperscript{b} Note prf-R is in 5’ to 3’ orientation in alignment. Actual primer sequence is the reverse complement.
Figure 5.5  Real-time PCR amplification of prf-specific product using the developed primer-and-probe set in testing common foodborne bacteria. ▼V7, ■ ScottA, ♦F2365, meat isolates □8517, ▲8576, +8578, ● 8732; Bacteria that gave no response (horizontal lines) are Pseudomonas putida ATCC49451, Escherichia coli DH5α, Listeria innocua and Lactococcus lactis 2301. CF RFU: curve fit relative fluorescence units.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence(^a)</th>
<th>Length</th>
<th>(T_m)(^c)</th>
<th>G+C content(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hly -FP</td>
<td>AGTCCTAAGACGCCAATC</td>
<td>18 bp</td>
<td>58.4°C</td>
<td>50%</td>
</tr>
<tr>
<td>hly -RP</td>
<td>TAACCTTTTTCTTGGCGGC(^b)</td>
<td>18bp</td>
<td>58.4°C</td>
<td>50%</td>
</tr>
<tr>
<td>hly-Probe</td>
<td>CCACGGAGATGCAGTGA</td>
<td>18bp</td>
<td>62.96°C</td>
<td>61.11%</td>
</tr>
<tr>
<td>prfA -FP</td>
<td>AAACTCCTGATGGCATC</td>
<td>17bp</td>
<td>55.6°C</td>
<td>47.06%</td>
</tr>
<tr>
<td>prfA -RP</td>
<td>CACGATAACTTTCTTTGC(^b)</td>
<td>19bp</td>
<td>54.9°C</td>
<td>42.11%</td>
</tr>
<tr>
<td>prfA-Probe</td>
<td>TCGCACATAGCTCAGCTG</td>
<td>18bp</td>
<td>59.2°C</td>
<td>50%</td>
</tr>
</tbody>
</table>

Table 5.1. Oligonucleotide data for *L. monocytogenes* probes and primers.

\(^a\) Sequences are in 5’ to 3’ direction  
\(^b\) Sequence shown is the reverse complement of the 5’ to 3’ sequence  
\(^c\) The melting temperature of the oligonucleotide as calculated by the manufacturer  
\(^d\) Percentage of guanine and cytosine nucleotides in the sequence
CHAPTER 6

HIGH-FREQUENCY CONJUGATION SYSTEM FACILITATES BIOFILM FORMATION AND PAMβ1 TRANSMISSION BY LACTOCOCCUS LACTIS

6.1 Abstract

The importance of conjugation as a mechanism to spread biofilm determinants among microbial population was illustrated with the Gram-positive bacterium Lactococcus lactis. Conjugation triggered the enhanced expression of the clumping protein CluA, which is a main biofilm attribute in lactococci. Clumping transconjugants further transmitted the biofilm-forming elements among the lactococcal population at a much higher frequency than the parental nonclumping donor. This cell-clumping associated high-frequency conjugation system also appeared to serve as an internal enhancer facilitating the dissemination of the broad-host-range drug-resistance gene-encoding plasmid pAMβ1 within L. lactis, at frequencies more than 10,000 times higher than the nonclumping parental donor strain. The implication of this finding to antibiotic resistance gene dissemination is discussed.
6.2 Introduction

Lactococci are important fermentation starter cultures. As commensal organisms, they are widely distributed (Heilig et al., 2002; Salama et al., 1993) and can be found co-existing with many other organisms, including pathogens, in natural and food processing environments, most commonly in the form of mixed culture ecosystems. However, to date, information regarding lactococcal biofilm formation is very limited (Mercier et al., 2002). The contribution of commensal organisms in mixed culture biofilm development involving pathogens and other risks associated with these biofilms have not been fully explored.

Lactococci are susceptible to various gene transfer mechanisms (Gasson 1990). Many important traits, including lactose utilization, proteolytic system, bacterial phage resistance, and nisin production and immunity, are associated with mobile elements. Of particular interest, a cell-clumping associated high frequency conjugal gene transfer system has been reported in two similar settings involving Lactococcus lactis strains ML3 and 712. In both cases, the conjugative elements, being the sex factor in 712 and pRS01 in ML3, mobilized the transfer of the Lac plasmid to the recipient cells by forming plasmid co-integrates. Some of the conjugation progenies exhibited cell auto-aggregation. When these clumping cells served as donors in the second round of mating, they transferred the Lac plasmid $10^2$-$10^7$ times more efficiently than the original donor strain (Anderson et al., 1984; Gasson and Davies 1980a; Walsh and McKay 1981). Divalent ions were required for auto-aggregation, and proteinase treatments significantly decreased both cell clumping (Clu$^+$) and high frequency conjugation (Wang et al., 1994). Strain MG1363 is a non-clumping, plasmid-cured derivative from strain 712 with the sex
factor retained in the chromosome. A cluA gene, which encoded a putative cell surface protein containing the well-conserved hexapeptide LPXTGE, was cloned from the sex factor. Expression of the CluA through upstream fusion of a lactococcal heat-shock promoter in MG1363 partially restored cell aggregation (Gordon et al., 1994).

It is anticipated that the biofilm environment is suitable for gene transfer events such as conjugation and transformation. However, a two-way relationship was recently demonstrated in *Escherichia coli*, showing that conjugation also served as an important mechanism for biofilm development, independent from quorum sensing (Vidal et al., 1998; Ghigo 2001; Molin and Tolker-Nielsen 2003; Reisner et al., 2003). Meanwhile, bacterial surface ligand-and-receptor interaction-mediated cell aggregation was found essential in oral bacteria biofilm assembly (Kolenbrander 1998; Kolenbrander 2000; Rickard et al., 2003). Therefore it is of particular interest to see whether there is a correlation between cell aggregation, high frequency conjugation, and biofilm formation in lactococci.

The main objective for this study was to examine the possible relationship between conjugation and biofilm development in *L. lactis*. Since cell aggregation associated high frequency conjugation systems have been reported in other organisms including *Bacillus thuringiensis* (Andrup et al., 1993; Jesen et al., 1995), *Lactobacillus plantarum* (Reniero et al., 1992), and *Enterococcus faecalis* (Dunny et al., 1978), revealing such a biofilm-forming mechanism may have broader implications. A hidden risk associated with organisms carrying such inherited high frequency conjugation systems is also discussed.
6.3 Materials and methods

6.3.1 Bacterial strains, growth conditions and plasmids

Bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1. Bacterial strains were stored at 4°C and maintained by biweekly transfers in appropriate media. Lactococcal strains were grown in M17 broth containing 0.5% glucose or lactose (M17-G or M17-L, Becton Dickinson and Company, Sparks, MD) as the sole carbohydrate source at 30°C. The final concentrations of antibiotics used for screening *L. lactis* derivatives were: 500 µg/ml streptomycin sulfate (Fisher Scientific, Fair Lawn, NJ), 5 µg/ml erythromycin (Fisher Scientific), and 5 µg/ml tetracycline (Sigma-Aldrich Co., St. Louis, MO). *Escherichia coli* strains DH-5α (Invitrogen, Carlsbad, CA) and INVαF’ (Invitrogen) were propagated in Miller LB broth (Fisher Scientific) at 37°C with aeration.

6.3.2 Mutant isolation

The tetracycline-resistant (Te') mutant HL01Te was isolated following procedures as described by McKay et al. (McKay et al., 1980). Basically, 5 ml of fresh M17-G broth containing 0.5 µg/ml of tetracycline was inoculated with 250 µl of an overnight culture of *L. lactis* LM2301 and incubated at 30°C for 2 to 3 days until growth was evident. Successive transfers were then made into M17-G broth with tetracycline concentrations of 2, 5 and 10 µg/ml, consecutively. The isolated mutant was maintained in M17-G broth containing 5 µg/ml of tetracycline.
6.3.3 DNA manipulation

For bacterial genomic DNA extraction, cells were collected from 1 ml of overnight culture by micro-centrifugation at 5400 ×g for 10 min. Collected cells were treated with 20 mg/ml of lysozyme (Sigma-Aldrich Co.) in enzymatic lysis buffer (20 mM Tris.Cl, pH 8.0, 2 mM EDTA, and 1.2% Triton X-100) for 45 min at 37°C. Genomic DNA was extracted using a commercial isolation kit (DNaseasy Tissue Kit; Qiagen, Valencia, CA) and eluted with 100 µl of elution buffer following the manufacturer’s instructions.

A 4133 bp cluA fragment, including the 3729 bp structure gene, 301 bp upstream and 103 bp downstream sequences, was amplified by conventional PCR using primer pair 5’CAGCTGTGGTTGATTCAAC3’ and 5’GATATCAATAAGGTAATGAG 3’, and the genomic DNA from MG1363 as the template. PCR products were purified using a commercial purification kit (QIAquick; Qiagen). Purified PCR products were cloned into the pCR 2.1 vector and the recombinant plasmid was transformed into E. coli INVαF’ competent cells using a cloning kit (TA Cloning; Invitrogen). The recombinant plasmid pCRCluA was recovered from the ampicillin-resistant transformant. A second PCR primer pair, 5’CGCGGATCCGTCTGATAAGGCAGTTTTTTTGT TTC3’ and 5’TAATACGACTCAGTATAGGG3’, was used to amplify a second 3.9 kb cluA fragment, using the above recombinant plasmid pCRCluA as the template. This new PCR amplicon contained the 3729 bp cluA structure gene and 48 bp upstream and 103 bp downstream sequences from MG1363, as well as flanking sequences from the vector pCR2.1. The fragment was digested with BamHI and XbaI, cloned into pMSP3535, and electroporated into E.coli DH5α. The transformants were screened on the BHI agar plate
containing 100 µg/ml of erythromycin. The recombinant plasmid pMSP3535CluA was recovered from \textit{E. coli} using a miniprep kit (QIAprep®; Qiagen). The DNA sequence of the cloned \textit{cluA} gene was partially confirmed using a DNA analyzer (ABI PRISM 3700®, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility of the Ohio State University. The recombinant plasmid pMSP3535CluA and the vector pMSP3535 were electroporated into \textit{L. lactis} strains, respectively, following the procedures described previously (Walsh and McKay 1981).

6.3.4 Expression of CluA

Nisin stock solutions were prepared by dissolving nisin (2.5% in milk solids, wt/wt. Sigma-Aldrich Co., catalog number N-5764-5G) in sterilized, distilled water to a final concentration containing 4 µg/ml nisin. The stock solution was stored at 4°C for up to one week. M17 broth supplemented with 5 µg/ml of erythromycin and appropriate carbohydrate was inoculated with the overnight culture of HL2301A or HL3A (5% of the final volume), and incubated at 30°C for 3 h. To induce CluA expression, nisin stock solution was added to the above cultures to a final nisin concentration of 25 ng/ml. The cultures were then incubated at 30°C for additional 15 h (making the total incubation time 18 h) for cell aggregation observation, or 33 h (making the total incubation time 36 h) for microtiter plate attachment assay, confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) analysis. \textit{L. lactis} strains HL2301V and HL3V were used as vector controls following the nisin induction procedures as described above. Furthermore, a parallel set of HL2301A and HL3A were incubated as CluA-expression
controls under the same conditions for the same period of time, except without adding nisin for induction.

6.3.5 RT-PCR

Five ml of M17-G or M17-L broth was inoculated with 250 µl of the overnight L. lactis cultures, and incubated at 30°C for 6 h until O.D.600 reached 0.8. RNA was extracted from 0.5 ml of such cultures using a commercial RNA extraction kit (RNeasy®; Qiagen). RNA was eluted by 50 µl of Rnase-free water and treated with amplification grade Dnase I (Invitrogen), following the instructions from the manufacturers. The pair of primers 5’ATGAAAAAAACATTGAGAGACCAG3’ and 5’ AAGTCCTGTCATTCCGTCG 3’ amplified a 636 bp fragment of the cluA (GenBank accession number U04468) gene. The expression of the L. lactis ribosomal protein L4, (GenBank accession number AE006438) was included as an internal standard. The primer pairs 5’CATGGCGTCAAAAAGG3’ and 5’ TGCAAGAACCTCCTCCTC3’ amplified a 434 bp region of the rl4 gene. The set up for RT-PCR in 50 µl reaction mixture contained: 25 µl of 2× SuperScript™ one-step RT-PCR reaction buffer (a buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO4, Invitrogen); 0.2 µM of each of the four primers; 1 µl of SuperScript™ II RT/Platinum® Taq Mix (Invitrogen); and 5 µl of the treated RNA sample. The RT-PCR cycling conditions and product gel electrophoresis followed standard procedures. The gel image was captured using a Bio-Rad Quantity One Gel doc system (Bio-Rad, Hercules, CA). Two units of Platinum® Taq DNA
polymerase (Invitrogen) instead of the RT/Platinum® Taq Mix were used in the RT-PCR controls to ensure the absence of DNA contaminations.

6.3.6 Cell aggregation

To aid in visualization of aggregation, cells from 10 ml of overnight culture of *L. lactis* strains with or without nisin induction were collected by centrifugation at 4,300 × g for 10 min. The cell pellets were resuspended in same volume of 0.85% saline and vortexed at maximum speed for 1 min. Non-clumping strains formed uniform solutions after resuspension. Within minutes clumping strains formed aggregates that can be observed visually.

6.3.7 Biofilm cultivation and evaluation

A rapid microtiter plate adherence test (Deighton, *et al.*, 2001) was conducted with modification to measure bacterial attachment on the surface. Each well containing 2 ml of appropriate M17 broth (24-well microtiter plate, Becton Dickison and Company) was inoculated with 100 µl of an overnight *L. lactis* culture and incubated at 30°C for 36 h. After incubation, 500 µl of 0.21% crystal violet staining solution (Fisher Diagnostic, Middletown, PA) was added to each well and incubated at room temperature for 10 min. Microtiter plate wells were rinsed with 2 ml distilled water to remove unattached cells and residual dye. The images of attached cells were captured by a Bio-Rad Quantity One Gel Doc system (Bio-Rad).

CLSM was used to examine *L. lactis* strains’ biofilm-forming potentials on Lab-Tek Permanox Plastic Chamber Slides (Nalgene Nune International corp., Naperville, IL).
Each chamber was filled with 1 ml of either M17-G or M17-L broth with appropriate antibiotics, and was inoculated with 50 µl of an overnight *L. lactis* culture. The slide was incubated at 30°C for 36 h, in the presence or absence of nisin. A chamber containing 1 ml of uninoculated broth was used as a medium control. Unattached cells and culture medium were washed off using 2 ml phosphate buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na$_2$HPO$_4$, 0.24 g KH$_2$PO$_4$, L$^{-1}$, pH 7.4) after the incubation. Surface-attached cells were fixed with 1% formaldehyde in phosphate buffered saline for 20 minutes at 4°C and then stained with 0.01% acridine orange (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature. The acridine orange was poured off and excess stain was removed by washing the slides one time with 2 ml phosphate buffered saline. After slides were mounted with cover slips, samples were observed using a confocal laser scanning microscope [Model LSM 510 META, Carl Zeiss, Inc., Thornwood, NY]. Hene1 laser at 543nm was used for excitation and Long Path LP560nm was used for emission filter. The biofilms were examined with a 63× oil immersion objective lens and the images were collected and analyzed using the packaged LSM 510 imaging software.

SEM was used to observe *L. lactis* biofilm structures. Lactococcal biofilms were cultivated by inoculating 100 µl of an overnight *L. lactis* culture into 2 ml of M17-G or M17-L broth per well in 24-well microtiter plates (Becton Dickison and Company), and incubated at 30°C, at the presence or absence of nisin. SEM sample preparation was conducted based on procedures as described by Li et al. 2001 and Marsh et al. 2003 with modification. Basically, biofilms formed on the surfaces of the microtiter plate wells were washed once with 2 ml of phosphate buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na$_2$HPO$_4$, 0.24 g KH$_2$PO$_4$, L$^{-1}$, pH 7.4) and fixed with 2 ml of 3.7% formaldehyde in...
phosphate buffered saline for 24 h at room temperature. The samples were then dehydrated by a graded 100% ethanol:hexamethyldisilazane (Ted Pella, Inc., Redding, CA) series (3:1 for 15 min, 1:1 for 15 min, 1:3 for 15 min, 100% hexamethyldisilazane 3×15 min), and left covered by hexamethyldisilazaneto dehydrate for overnight. After dehydration, the bottom surface of the well was cut off, mounted and sputter-coated for 180 s with gold palladium (Pelco Model 3 Sputter Coater, Ted Pella, Inc., Redding, CA). Samples were observed using a scanning electron microscope (FEI XL30, FEI Company, Hillsboro, OR). Images were captured at low magnification for biofilm network overview and high magnification to show detailed biofilm structure.

6.3.8 Conjugal matings

Conjugal matings were performed using the direct plating method described previously with modification (Wang et al., 1994). Cells from 1.7 ml of 18 h cultures, including strains HL2301V, HL2301A, HL3V, HL3A with nisin induction, and HL2301, HL3A without nisin induction, were harvested by centrifugation at 5,400 \( \times \) g for 5 min. The cell pellets were resuspended in 5 ml of fresh medium and inoculated for 1.5 h at 30°C before harvesting. For the rest of the strains, 0.1 ml of an overnight culture was inoculated into 5 ml of M17 broth and incubated at 30°C for 4 h (O.D.\textsubscript{600} to around 0.6, approximately \( 10^8 \) to \( 10^9 \) cfu/ml). Cells were then harvested by centrifugation at 4,300 \( \times \) g for 10 min, and resuspended in 0.5 ml of saline. Donor and recipient cells were mixed at a 1:2 ratio and 0.2 ml of the cell mixture or its dilution was plated directly on BCP-Lactose indicator agar (McKay et al., 1972) that contained 500 µg/ml of streptomycin sulfate, 5 µg/ml of erythromycin, or 5 µg/ml of tetracycline. Lactose-positive
transconjugants were verified by phenotypic characterization. BCP-Glucose indicator agar that contained 5 µg/ml of erythromycin and 5 µg/ml of tetracycline was used to screen for transconjugants containing pAMβ1, using HW401 as donor and HL01Te as recipient. Transfer frequencies were expressed as the number of transconjugants per colony-forming unit of the donor, and the values reported were the mean values from three separate experiments.

6.4 Results

6.4.1 The involvement of conjugation in lactococcal biofilm development

The transmission of Lac plasmid among L. lactis strains is illustrated in Fig.1. Biofilm formations by the initial donor ML3, the recipient LM2301, and the Clu⁺ transconjugant HW002 were examined. The Clu⁺ strain HW002 was found to be more efficient in forming biofilms than both the non-clumping donor ML3 and the recipient LM2301. Thick, three-dimensional biofilm structure by the Clu⁺ strain HW002, after 36 h incubation at 30°C, was evident by SEM. However, under the same conditions, the parental strains ML3 and LM2301 only developed attachment on limited surface areas with mostly single layer of cells (Fig. 6.2A-F). In the subsequent matings, the plasmids were transferred from HW002 to various lactococcal recipients at high frequencies (Fig.6.1). The majority of these transconjugants exhibited Clu⁺ phenotype. As expected, clumping strains HL221, HL331, HW401, HL421, HL332 and HL222 all exhibited enhanced biofilm-forming ability (Fig. 6.3). These results indicated that cell aggregation and enhanced biofilm formation were correlated in L. lactis, and that these phenotypes
were transferable by conjugation. The Clu<sup>+</sup> strains could transfer the plasmids at frequencies 10<sup>2</sup>-10<sup>7</sup> times higher than the non-clumping donor, and were therefore much more efficient in disseminating the biofilm-forming attributes among the population than the initial parental strain.

While ML3 contained multiple plasmids and was the donor for the Lac plasmid pSK08 and the conjugative plasmid pRS01 in the conjugal mating, it was a much slower biofilm former and did not form cell clumping. A logical interpretation would be that the plasmid-recombination event during co-integrate formation triggered the expression of certain protein(s), which was involved in biofilm development. To test this hypothesis, the expression of CluA in ML3, LM2301 and the clumping transconjugant HW002 were compared. As expected, the transcription of cluA gene in HW002 was found to be much higher than that in ML3, and no transcription was observed in the plasmid-cured recipient strain LM2301 by RT-PCR (Fig. 6.4). To further confirm the role of CluA in lactococcal biofilm formation, the cluA gene was cloned into the expression vector pMSP3535, downstream of the nisA promoter. The expression of CluA protein was induced by external nisin signal and confirmed by SDS-PAGE (data not shown). Strains HL3A and HL2301A both exhibited cell aggregation with nisin induction. SEM study showed that both strains also exhibited enhanced biofilm formation with nisin induction rather than without (Fig. 6.5 and Fig.6.6). The biofilm formed by CluA-expressed HL3A with nisin induction was 7-8 times thicker than that by the same strain without induction (Fig. 6.7). However, although clumping HL2301A developed localized biofilm structure with nisin induction, which was absent by the same strain without the inducer (Fig. 6.6A-B), its structure was much less complex than that resulting from the clumping HL3A (Fig. 6.5A-
B) and HW002 (Fig. 6.2E-F). These results suggested that CluA was the clumping factor as well as a key biofilm determinant. Increased expression of CluA facilitated biofilm formation, which was triggered by the conjugation event in *L. lactis*. Nevertheless, ML3 might carry additional plasmid-encoded element(s), other than CluA, which also contributed to biofilm structure development.

### 6.4.2 Role of CluA in conjugal gene transfer

It was anticipated that the high frequency conjugal gene transfer ability as illustrated by Clu\(^+\) strains, such as HW002, was largely due to the close proximity between the donor and recipient cells within cell aggregates. Therefore experiments were conducted to examine the role of CluA expression in conjugal gene transfer using the direct plate conjugation method. The expression of CluA was induced either in donor or recipient, and the frequencies of conjugal transfer of the Lac plasmid were compared. As illustrated in Table 6.2, the conjugation frequency between the non-clumping donor ML3 and the clumping recipient HL2301A with nisin induction was significantly higher than the frequency by the control setting without nisin induction (P<0.05); the difference between the mean values was about 3-fold. Likewise, the frequency of conjugation between the clumping donor HL3A with nisin induction and LM2301 was significantly higher than the control setting without nisin induction (P<0.05); the difference in mean values was about 8-fold. These results suggested that the expression of CluA enhanced the conjugal gene transfer event, regardless of whether the CluA expression was associated with donor or recipient. However, induced expression of CluA in donor or recipient did not restore high frequency conjugation (10\(^2\) to 10\(^7\) times higher than the
parental strains) exhibited by some of the clumping transconjugants. The contribution of cell aggregation to high frequency conjugation was rather limited, indicating the involvement of additional factor(s) in such high frequency conjugal gene transfer events. This is in agreement with a proposed conjugal gene transfer model that at least two protein complexes are required, involving DNA preparation and mating-pair formation (Grohmann et al., 2003). The lactococcal-inherited system should carry all the essential elements required for high frequency gene transfer. CluA is likely involved in mating pair formation.

6.4.3 Facilitated transfer of pAMβ1 by the lactococcal high frequency conjugation system

While the lactococcal high frequency conjugation system was known for the transfer of beneficial fermentation traits, its potential involvement in antibiotic resistance gene transmission was investigated. As illustrated in Fig. 6.1, HW002 served as a “super donor”, transferring the Lac plasmid in the second round of mating at frequencies up to $10^{-2}$ transconjugant/per donor cfu, about $10^6$ times more efficient than that by the parental strain. Although the major contributor(s) for the high frequency conjugation system is yet to be identified, the potential role of such intrinsic mechanism in transmitting the antibiotic resistance genes was examined (Fig. 6.8). The direct transfer of pAMβ1 from the donor strain JK2301β to the recipient HL01Te was a low efficiency event (Fig. 8A). However, when JK2301β mated with HW002, a pool of clumping “super donor II” transconjugants containing pAMβ1 was generated at $10^{-2}$ transconjugant/donor CFU (Fig.
6.8B). These “super donor II” cells (HW401-like) further transferred pAMβ1 to the HL01Te at frequencies 10,000 times higher than the original donor JK2301β (Fig. 6.8C).

6.5 Discussion and Conclusion

The relationship between gene transfer and biofilm is of great interest to the scientific community. In this study, we have demonstrated that in L. lactis, the sex factor encoded cell surface component CluA is the key factor in L. lactis cell aggregation and biofilm development; enhanced expression of CluA and the subsequently facilitated biofilm-formation is the direct result of a conjugation event. Furthermore, this enhanced biofilm-forming trait is transmissible by conjugation. Evidences in the past showed that gene transfer might be facilitated in cell clumps or in the biofilm environment through a better donor-recipient interaction. Our study is significant because it illustrates a new relationship between conjugation and biofilms in Gram-positive bacteria: gene transfer events such as conjugation further enable these organisms to disseminate biofilm attributes effectively within the community to enhance the ecosystem development. This result is consistent with the discovery in E. coli that conjugation is an independent mechanism for biofilm development.

It is anticipated that cell clumping is directly correlated to high frequency conjugation. However, while nisin-induced expression of CluA, regardless in donor or recipient, increased conjugation frequency, the scale of improvement is much less than expected. Conjugation frequencies by mating sets C and F (Table 6.2) clearly showed that cell clumping is not the major contributor for high frequency gene transfer in
lactococci. Limited biofilm enhancement by strain HL01A with nisin induction (Fig. 6.6A, 6.6B) further suggested that other plasmid-encoded factor(s) were involved in facilitating the development of a well-connected biofilm network as exhibited by the clumping strains (Fig. 6.2E, 6.2F). Characterization of these additional factors involved in both the high frequency conjugal gene transfer and enhanced biofilm formation is currently under way.

Increased pathogen resistance to antibiotic treatments is a major threat to human health. The roles of commensals, especially foodborne microbes in transmitting resistance genes are becoming a recent concern to the scientific community (Andremont 2003). In the past, various mobile elements encoding antibiotic resistance determinants had been identified and characterized in both foodborne pathogens and commensals isolated from foods, animals and humans (Levy et al., 1976; Goldstein et al., 2001; Blake et al., 2003; Gever et al., 2003; Chen et al., 2004; Nandi et al., 2004). These data suggest that food can be an important carrier introducing antibiotic resistance genes into humans, and that antibiotic-resistant pathogens and commensals have emerged in the food chain. However, the picture is still incomplete.

The broad-host range, erythromycin resistance gene encoding plasmid pAMβ1 was initially identified in E. faecalis (Clewell et al., 1974). The transmission of this plasmid between E. faecalis, L. lactis, and Lactobacillus spp. strains by conjugation was documented (Gasson and Davies 1980b; Vescovo et al., 1983; Tannock 1987; Pucci et al., 1988). Therefore monitoring the transfer of this plasmid in ecosystems has practical implications in understanding the transmission of antibiotic resistance genes among commensals and pathogens.
In this study, using *L. lactis* as a model organism, we have demonstrated two concepts: first, a pool of clumping lactococal progenies with the broad-host range erythromycin-resistance gene-encoding plasmid pAMβ1 can be generated very efficiently by strains carrying the intrinsic high frequency conjugation mechanism (Fig. 6.8B); second, this inherited mechanism, previously known for the transfer of fermentation traits such as lactose utilization and the proteolytic system, can serve as an enhancer facilitating the subsequent transfer of pAMβ1 among lactococcal population (Fig. 6.8C). Therefore, we have demonstrated that organisms carrying such intrinsic mechanisms not only have the potential to become an important reservoir for antibiotic resistance genes, but more importantly, that these intermediate organisms can disseminate antibiotic resistance genes (at least pAMβ1) in subsequent events much more effectively than the parental donor strain. Currently, these concepts are illustrated in lactococci, which are found mostly in the dairy fermentation environment. However, inherited high frequency conjugation systems have also been reported in several other bacteria including lactobacilli, enterococci and bacilli, which are commonly found in food production and processing environments, as well as in animal and human host ecosystems. Some of these organisms are consumed in large quantities as probiotics, while screening of the presence of inherited gene-transfer mechanisms is not routinely conducted. It is therefore of great interest to investigate whether such “enhancing” mechanisms also function similarly in these organisms, and what kind of roles these organisms, particularly commensals, could have played in disseminating antibiotic resistance and other microbial “surviving friendly” genes, in both environment and hosts.
6.6 References


Figure 6.1. Conjugal transfer of the Lac plasmid among *L. lactis* strains. *The clumping transconjugants served as “super donors”, transferred the Lac plasmid at $10^{-2} - 10^{-3}$ transconjugant/donor cfu in subsequent matings.*
Figure 6.2. SEM pictures of 36 h biofilms by *L. lactis* strains involved in conjugation. A: Overview of the biofilm formed by the donor strain ML3. B: Regional magnification of A showing biofilm details. C: Overview of the biofilm by the recipient strain LM2301. D: Regional magnification of C showing biofilm details. E: Overview of the biofilm by the clumping transconjugant HW002. F: Regional magnification of E showing biofilm details. The bars in A, C, and E represent 50µm; the bars in B, D, and F represent 5µm.
Figure 6.3. Biofilm formation and the conjugal transfer of enhanced biofilm-forming phenotype by *L. lactis* clumping strains using the rapid microtiter plate adherence test. ML3 was the initial donor for the Lac plasmid. LM2301, JK2301β, LM2302, HL01Te were conjugation recipients. HW002, HL221, HL331 and HW401 were clumping transconjugants, and they also served as donors for the Lac plasmid in the subsequent matings. HL421, HL332, and HL222 were clumping transconjugants.

Figure 6.4. Comparison of the CluA expression in *L. lactis* strains by RT-PCR. Lane M: 100-bp DNA ladder. Lane 1: HW002. Lane 2: ML3. Lane 3: LM2301. Lane 4 through 6 are DNA amplification controls using the same RNA samples of HW002, ML3 and LM2301, respectively, but without reverse transcriptase. The arrows indicate the positions of the 434 bp internal standard *rl4* gene amplicons, and the 636 bp *cluA* gene amplification products.
Figure 6.5. SEM pictures of biofilm formation by *L. lactis* HL3A. A: With nisin induction. B: Regional magnification of A showing biofilm details. C: Without nisin induction. D Regional magnification of C showing attachment details. The bars in A and C represent 50 µm, and in B and D represent 2 µm.
Figure 6.6. SEM pictures of biofilm formation by *L. lactis* HL2301A. A: With nisin induction. B: Without nisin induction. The bars represent 5μm.
Figure 6.7. CLSM pictures of biofilm formation by *L. lactis* HL3A. A: With nisin induction. B: Without nisin induction. Top panels are the xz plane, and the bottom panels are the xy plane. The bars represent 5 µm.
Figure 6.8. Conjugal transfer of plasmid pAMβ1 among *L. lactis* strains. A: JK2301β transferred the plasmid pAMβ1 to the recipient HL01Te at a frequency of $10^{-8}$ transconjugant/donor cfu. B: Conjugation between JK2301β and the clumping strain HW002 generated the clumping, Lac<sup>+</sup> Em<sup>r</sup> transconjugants (HW401-like) at the frequency of $10^{-2}$ transconjugant/donor cfu. C: HW401 transferred pAMβ1 to HL01Te at the frequency of $10^{-4}$ transconjugant/donor cfu.
## Table 6.1 Bacterial strains and plasmids used in the study

<table>
<thead>
<tr>
<th>Strain and plasmid</th>
<th>Relevant phenotype$^1$ Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCRCluA</td>
<td>Amp$^d$ CluA cloned in pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>pMSP3535</td>
<td>Em$^t$ Nisin inducible expression vector</td>
<td>Bryan et al., 2000</td>
</tr>
<tr>
<td>p3535CluA</td>
<td>Em$^t$CluA$^t$ pMSP 3535 with cloned cluA, nisin inducible</td>
<td>this study</td>
</tr>
<tr>
<td>pAMβ1</td>
<td>Em$^t$ Broad-host-range drug-resistant plasmid from E. faecalis</td>
<td>Clewell et al., 1974</td>
</tr>
<tr>
<td>L. lactis ML3 (104, 55, 48.4, 8.5, 3.0, 1.5)</td>
<td>Lac$^{-}$Clu$^{-}$Str$^{-}$Em$^{-}$</td>
<td>Parental Lac$^{-}$ donor</td>
</tr>
<tr>
<td>L. lactis MG1363</td>
<td>Lac$^+$Clu$^+$</td>
<td>plasmid cured derivative of 712</td>
</tr>
<tr>
<td>L. lactis LM2301</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>Plasmid-cured, Str$^+$ derivative of L. lactis strain C2</td>
</tr>
<tr>
<td>L. lactis LM2302</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>Plasmid-cured, Em$^t$ derivative of LM2301</td>
</tr>
<tr>
<td>L. lactis HW002</td>
<td>Lac$^+$Clu$^+$Str$^+$Em$^t$</td>
<td>Clumping transconjugant from ML3 x LM2301</td>
</tr>
<tr>
<td>L. lactis HL2301A</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>LM2301 transformed with p3535CluA</td>
</tr>
<tr>
<td>L. lactis HL2301V</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>LM2301 transformed with pMSP3535</td>
</tr>
<tr>
<td>L. lactis HL3A</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>ML3 transformed with p3535CluA</td>
</tr>
<tr>
<td>L. lactis HL3V</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>ML3 transformed with pMSP3535</td>
</tr>
<tr>
<td>L. lactis HL01Te</td>
<td>Lac$^+$Str$^+$Em$^t$Te$^t$ Spontaneously 2301 mutant, tetracycline resistant</td>
<td>this study</td>
</tr>
<tr>
<td>L. lactis JK2301β</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>Transformant of LM2301 received pAMβ1</td>
</tr>
<tr>
<td>L. lactis HW401</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>Clumping transconjugant from HW002 x JK2301β</td>
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<tr>
<td>L. lactis HL421</td>
<td>Lac$^+$Str$^+$Em$^t$Te$^t$</td>
<td>Clumping transconjugant from HW401 x HL01Te</td>
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<td>L. lactis HL221</td>
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<td>Clumping transconjugant from HW002 x HL01Te</td>
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<tr>
<td>L. lactis HL332</td>
<td>Lac$^+$Str$^+$Em$^t$</td>
<td>Clumping transconjugant from HL331 x LM2302</td>
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</tbody>
</table>

$^1$Abbreviations: Clu, ability (+) or inability (-) to self-aggregate; Amp, ampicillin-resistant (r) or -sensitive (s); Str, streptomycin-resistant (r) or -sensitive (s); Em, erythromycin-resistant (r) or -sensitive (s); Te, tetracycline-resistant (r) or -sensitive (s); Lac, ability (+) or inability (-) to ferment lactose.
<table>
<thead>
<tr>
<th>Set</th>
<th>Donor</th>
<th>Recipient</th>
<th>Nisin induction</th>
<th>Conjugation frequency</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ML3</td>
<td>LM2301</td>
<td>N/A</td>
<td>$1.5 \times 10^{-8} \pm 6 \times 10^{-9}$</td>
<td>Original setting</td>
</tr>
<tr>
<td>B</td>
<td>HW002</td>
<td>LM2302</td>
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<td>$2.5 \times 10^{-2} \pm 7 \times 10^{-3}$</td>
<td>“Super donor I”</td>
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<td>C</td>
<td>ML3</td>
<td>HL2301A</td>
<td>Yes</td>
<td>$7.2 \times 10^{-8} \pm 2.9 \times 10^{-8}$</td>
<td>CluA induced in recipient</td>
</tr>
<tr>
<td>D</td>
<td>ML3</td>
<td>HL2301A</td>
<td>No</td>
<td>$2.1 \times 10^{-9} \pm 1.9 \times 10^{-9}$</td>
<td>Control without CluA induction</td>
</tr>
<tr>
<td>E</td>
<td>ML3</td>
<td>HL2301V</td>
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<td>$2.6 \times 10^{-8} \pm 1.8 \times 10^{-8}$</td>
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</tr>
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<td>F</td>
<td>HL3A</td>
<td>LM2301</td>
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<td>$3.3 \times 10^{-7} \pm 2.0 \times 10^{-7}$</td>
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<td>HL3A</td>
<td>LM2301</td>
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<tr>
<td>H</td>
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<td>LM2301</td>
<td>Yes</td>
<td>$2.7 \times 10^{-8} \pm 1.1 \times 10^{-8}$</td>
<td>Control with cloning vector only</td>
</tr>
</tbody>
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Table 6.2 *Lactococcus lactis* conjugal mating

*All strains used in this table are derivatives of ML3 and C2, therefore all contain the Agg factor.*
7.1 Abstract

In food processing environments, bacteria are usually present in multi-species ecosystems, such as microbial biofilms, which are important sources for secondary contamination. Identification of commensal and pathogenic microorganisms in an ecosystem and characterization of their interactions can reveal the causes of pathogen persistence in the environment and factors that might affect biofilm formation. Certain biofilm forming bacteria may serve as backbone organisms in the ecosystems, and the prevalence of which may be directly related to the likelihood of pathogen persistence in the particular environments. The objectives of this part of work are to identify isolates from an existing ecosystem, and to examine the ability of dominant organism(s) in facilitating pathogen persistence by forming mixed culture biofilms. Microbiota from a laboratory ecosystem were analyzed by amplifying 16S rDNA fragments and sequencing the resulted amplicons. The dominant genera were *Staphylococcus* and *Pseudomonas*. The isolates of these genera were further evaluated for morphological properties and
their 16S-23S rDNA intergenic spacer regions (ISR) were sequenced. Two isolates, *Pseudomonas* sp. HL01 and *Staphylococcus* sp. HL02, as well as *Bacillus subtilis* IG-20 were used to cultivate single- and mixed-culture biofilms. The results showed that *Pseudomonas* sp. HL01 is a strong biofilm former by itself. The presence of such bacterium can facilitate ecosystem development involving other weaker biofilm forming strains, suggesting that even commensals could have contributed to the pathogen persistence in the environment. Therefore minimizing contamination by primary biofilm-forming strains, not just limited to pathogens, is critical for food safety.

### 7.2 Introduction

While evaluating the ability of a single bacterial culture attaching to abiotic surface and forming biofilm is important, recent evidence shows that the interactions among various microbes are as important in ecosystem development. Even if certain bacterial strains are weak biofilm formers by themselves, they might be able to be incorporated into an existing ecosystem, with the presence of proper partner organisms. Various signals and cell-cell interactions in the ecosystem can further alter gene expression patterns in these organisms for a better fitness. Therefore, identifying partner organisms in an ecosystem and characterization of the interactions between these organisms are important steps towards an advanced understanding of microbial persistent mechanism.

Among various approaches to identify microbial composition in complicated ecosystems, rRNA sequence analysis has become a popular tool in recent years (Woese 1987; Schönhuber *et al.*, 2001Botteger* et al.*, 1989; Edwards* et al.*, 1989; Weisburg* et al.*, 1989).
Most bacteria have multiple copies of the small ribosomal subunit RNA (16S rRNA) gene, also called 16S rDNA, which are highly conserved and share universal function and distribution among bacteria (Corless et al., 2000). 16S rRNA is a structural backbone of the ribosomal small subunit, and also works as an interaction site between the small and large subunits. 16S rRNA has a complicated looped secondary structure containing many helical regions, and it also has a critical binding site involved in the initiation of protein synthesis and may have function in decoding and elongation in protein synthesis (Brimacombe 1988). Because of the high conservation in all organisms and variation contained within the gene, 16S rDNA has been used extensively in determining taxonomic classifications and evolutionary relationships (Brimacombe 1988; Weisburg et al., 1991). The 16S rDNA is approximately 1500 bp and was first sequenced from *E.coli* in 1971, and since then 16S rDNA has been used as a good target for taxa identification and as a target for rapid microbial detection (Connor et al., 2005). Specific regions can be found within the 16S rDNA that are conserved at various levels, such as genus and species. Recently, the integenic spacer region (ISR) between 16S rDNA and 23S rDNA (Fig 7.1) has also been reported to fingerprint *E.coli*, *Bacillus* spp., *Porphyromonas gingivalis* to species or strain level, since it contains more variable regions than 16S rDNA (Leys et al., 1999, Daffonchio et al., 2003; Seurinck et al., 2003). During our investigation of the contribution of cell clumping to biofilm development, a microbial ecosystem was found in a test tube, where cell mixtures formed heavy coagulates quite resistant to vortex disruption. The objective of this study is therefore to identify key microbes in such an ecosystem and to evaluate the biofilm
forming abilities of these organisms and the potential role of such organisms in pathogen persistence.

7.3 Materials and methods

7.3.1 Bacterial strains and growth conditions

An aggregation sample was obtained from a liquid culture grown in Tryptic Soy Broth (TSB, Becton Dickinson and Company, Sparks, MD), 30°C. Single culture bacterial isolates from this sample were cultured in the same medium, incubated at 30°C for 18-24 hours. Bacillus subtilis IG-20 was obtained from the Bacillus Strain Collection Center, OSU. The strain was grown in Difco Nutrient Broth, at 37°C for 18 h. Frozen stocks of all strains were stored in their respective media, supplemented with 20% glycerol and kept at -80°C. Working cultures were kept at 4°C and maintained by biweekly transfers in corresponding media.

7.3.2 DNA extraction, rDNA cloning and sequencing

For genomic DNA extraction, cells were collected from 1 ml of bacterial culture by micro-centrifugation at 5400 ×g for 10 min. Collected cells were treated with 20 mg ml⁻¹ of lysozyme (Sigma Chemical CO., St Louis, MO) in enzymatic lysis buffer (20 mmol l⁻¹ Tris.Cl, pH 8.0, 2 mmol l⁻¹ EDTA, and 1.2% Triton X-100) for 45 min at 37°C. Genomic DNA was extracted using a commercial isolation kit (DNeasy Tissue Kit, Qiagen, Valencia, CA) and eluted with 100 μl of elution buffer following the instructions from the manufacturer. The 16S rDNA fragment was amplified using the 16S rDNA primer pair 16S rDNA-FP (5’AGAGTTTGATCCTGGCTCAG 3’), 16S rDNA-RP (5’
TACCTTGTTACGACTT 3’) (Edwards et al., 1989; Weisburg et al. 1991; Connor et al.,
2005) and the genomic DNA extracted from the sample. PCR conditions were: one cycle
at 95°C for three min, followed by 30 cycles at 95°C for 30 s, 50°C for one min and 72°C
for two min, with a final extension at 72°C for seven min using a thermal cycler
(iCycler™, Bio-Rad, Hercules, CA). PCR products were purified using a commercial
purification kit (QIAquick®, Qiagen, Valencia, CA) following manufacturer’s instruction.
Purified PCR products were cloned into pCR 2.1 vectors and transformed into E. coli
INVαF’ competent cells using a cloning kit (TA Cloning®, Invitrogen, Carlsbad, CA).
Recombinant plasmids of several randomly chosen colonies from the transformants
screening plate were recovered using a miniprep kit (QIAprep®, Qiagen, Valencia, CA).
The cloned 16SrDNA sequences in these plasmids were determined with T7 primer
(5’TAATACGACTCACTATAGGG 3’), using a DNA analyzer (ABI PRISM® 3700,
Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio
State University. The obtained sequences were compared with sequences from the
GenBank database, using a sequence comparison algorithm (Altschul et al., 1990).

The ISR fragment was amplified by PCR using the 785-FP 16S rDNA Universal
primer (5’GGATTAGATACCCTGGTAGTC3’), the 422-RP 23S rDNA Universal
primer (5’GGAGTATTTAGCTT3’) (Runf et al., 1999; Fig 7.1), and genomic DNA of
the isolates. PCR conditions were as described previously. The sequencing grade primers
of 785-FP and 422-RP were used to directly sequence the PCR products respectively. The
ISR sequences were compared with those from the GenBank database.
7.3.3 Isolation and confirmation of bacterial isolates from the sample

The environmental sample was also streaked on TSA plates and incubated at 30°C for 24 hours for single colony isolation. Thereafter, colony morphology comparison, Gram-staining test, cell morphology examination under light microscopy (American optical, Southbridge, MA) were conducted to some of the single colonies from the TSA plates. Representative colonies were chosen for further identification using ISR sequence analysis to further confirm the identification of the isolated single colonies.

7.3.4 Biofilm cultivation and evaluation

Wide-field microscopy (WFM) was used to examine biofilm-forming potentials on Lab-Tek Permanox Plastic Chamber Slides (Nalgene Nunc International corp., Naperville, IL). The strains were inoculated either individually at 2% or mixed at 1% each of overnight culture in 1 ml of TSB on each chamber. The slide was then incubated at 30°C for 6 h or 14 h. A chamber containing 1 ml of uninoculated broth was used as a medium control. After the incubation, the slides were washed: Unattached cells and culture medium were washed off using 30 ml phosphate buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, L⁻¹, pH 7.4) for 3 times after the incubation. Surface-attached cells were fixed with 1% formaldehyde in phosphate buffered saline for 20 minutes at 4°C and then stained with 0.01% acridine orange (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature. The acridine orange was poured off and excess stain was removed by washing the slides one time with 30 ml phosphate buffered saline. After slides were mounted with cover slips, the development of the attachment
patterns strains was monitored by microscopy with excitation wavelength of 475 nm and emission wavelength of 530 nm, 400× or 1000× magnification were used for the observation.

7.4 Results

7.4.1 Identification of bacteria from environmental sample

7.4.1.1 16S rDNA sequencing

The 16S rDNA fragments from the bacteria in the coaggregation ecosystem were PCR amplified, cloned into the vectors, and transformed into E. coli DH5α. The 16S rDNA inserts from plasmids extracted from representative transformants were sequenced. The 16S rDNA insertion of pHLE1 (Fig 7.2) was found belong to Pseudomonas. sp. (Godfrey et al., 2001), and with 98% sequence identity to P. fluorescens, P. jessenii and P. putida.

The 16S rDNA insertions of pHLE2 (Fig 7.3) has a highest sequence homology (99% identity) to an unknown strain of Staphylococcus sp. Its identities to S. paterui, S. aureus and S. epidemis were 99%, 98%, and 98%, respectively.

7.4.1.2. Colony identification

Two types of bacterial colonies with apparently different colony morphology were found on the TSA plates. Two representative colonies were picked. One was semi-transparent colony, and the other one was golden-yellow colony. By Gram-staining, the cells from the former one were gram-negative short-rods; and the cells from the later one were gram-positive cocci. ISR sequence analysis showed that the semi-transparent colony
has high homology to *Pseudomonas fluorescens* (97% identity) and *Pseudomonas putida* (97% identity). The golden-yellow colony has high homology to *Staphylococcus* sp. The two isolates were further named as *Pseudomonas* sp. HL01 and *Staphylococcus* sp. HL02.

### 7.4.2 Biofilm characteristics of isolates and *Bacillus subtilis IG-20*

The *Pseudomonas* sp. HL01 strain was found to be a strong biofilm former by itself (Fig.7.4b, 7.5b). *B. subtilis* IG-20 strain didn't attach to surfaces as examined by WFM after 6 h of incubation (Fig.7.4a). However, in the mixed culture situation, *B. subtilis* IG-20 was found attaching to the primary biofilm formed by *Pseudomonas* sp. HL01, and the mixed culture ecosystem was evident (Fig.7.4c). *Staphylococcus* sp. HL02 attached to surfaces slightly as examined by WFM after 14 h of incubation (Fig. 7.5a). However, in the mixed culture situation, *Staphylococcus* sp. HL02 was found attaching to the surface and formed biofilm to become a part of the ecosystem at the presence of *Pseudomonas*. sp. HL01 (Fig. 7.5c).

### 7.5 Discussion and Conclusion

16S rDNA have been used as a target gene for bacterial identification. In this study we used PCR amplification and 16S rRNA gene sequence analysis to identify bacteria from an environmental sample to genus level. The primers, 16S rDNA-FP: 5’AGAGTTTGATCCTGGCTCAG 3’ and 16S rDNA-RP: 5’ TACCTTGTTACGACTT 3’ for PCR amplification and direct sequencing were modified from Weisburg *et al.*, 1991 (Table 7.1): 16S rDNA-FP was exactly designed as the same as FP1 to target most
eubacteria, and 16S rDNA-RP was designed based on the common sequences of RP1, RP2 and RP3 in order to involve a more wide variety of bacterial taxa.

The strains were identified as *Pseudomonas* sp. and *Staphylococcus* sp. The results are in agreement with colony morphology, Gram-staining and cell morphology examinations. The identities of the isolates were confirmed by ISR sequencings, indicating that rDNA sequencing is a useful tool for bacterial identification in this study.

In this study, we examined the contribution of a mixed culture ecosystem in promoting the attachment of the so-called "non-biofilm-forming" organism in the environment. We demonstrated that *B. subtilis* IG-20, a "non-biofilm-former" by itself, could become part of the ecosystem involving *Pseudomonas* sp. HL01, a strong biofilm forming strain. We also demonstrated that *Staphylococcus* sp. HL02, a weak biofilm-former by itself, could form strong mixed culture biofilm at the presence of *Pseudomonas* sp. HL01. We therefore concluded that certain bacterial strains might be "non-biofilm-former" by themselves, but can attach and survive in the environment and become part of the ecosystem involving other "primary" biofilm-forming strains.

This study is significant because it illustrated that pathogenic or spoilage microorganisms could become persistent in the food processing environment even if they don’t form biofilm by themselves. Therefore investigation should not be limited to single strain biofilm forming abilities to evaluate the strains’ persistent potential. Minimizing contamination by primary biofilm-forming strains is critical for food safety.
7.6 References


Figure 7.1 Map of the ribosomal genes including the ISR and primer-binding positions. ISR, intergenic spacer region between 16S rDNA gene and 23S rDNA gene; rDNA, ribosomal DNA. (Modified from Runf et al., 1999)
AGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAA
AGGAGCTTGCTCTGGATTTCAGCAGGCAGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGT
GGGGGACAACGTTCGAAAGGAACGCTTAATACCCGCTACCTACGGGAGAAACAGGGGACCT
TCGGGCTTGCCCTACAGATGAGCCTAGGCAAGCTATAGCTTGGGAGTAATGGGCTCACCA
AGGCGACGATCCTGGTAACCTTGTGTAAGGATAGTACGTCACACTGGAACTGAGACACGGTCCAGA
CTTCTAACGGGAGGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGC
GTGTGGAAGAAGGTCTTTGGATTGTAAAGCATTTAAGTTGGGAGGAAGGCAGTAAATTAATA
CTTGTCTTTGAGCTTACCGACAGAAATAAGCACCAGCTAATCTGTGCAACGCGGCCGTA
TACAGAGGTCGCAAGCCTTAATCGGAATTACTGGGCTAAAGCGCGCGTAGGTGGTTAAGT
TGGATGTGAAATCCCGGCTCAACCTTGGAGCTGCACTCAGAAACTTGCAAGCTAGATATGGTA
GAGGTGTGGGAATTTTCTGTGTAACGGGAGAAATCGTAGATATTAGGAAGGAACACAGTGGCGA

Figure 7.2 16S rDNA gene sequence cloned in pHLE1
Figure 7.3 16S rDNA gene sequence cloned in pHLE2
Figure 7.4 Biofilm structure by individual or mixed bacteria. a. *B. subtilis* IG-20, 6 h; b. *Pseudomonas* sp. HL01, 6 h; c. *Pseudomonas* sp. HL01+ *B. subtilis* IG-20, 6 h.
Figure 7.5. Biofilm structure by individual or mixed bacteria. a. *Staphylococcus* sp. HL02 14 h; b. *Pseudomonas* sp. HL01, 14 h; c. *Pseudomonas* sp. HL01+ *Staphylococcus* sp. HL02, 14 h.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Designed for</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP1</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>Most eubacteria</td>
</tr>
<tr>
<td>FP2</td>
<td>AGAGTTTGATCATGGCTCAG</td>
<td>Enterics and relatives</td>
</tr>
<tr>
<td>FP3</td>
<td>AGAGTTTGATCCTGGCTTAG</td>
<td>Borrelia spirochetes</td>
</tr>
<tr>
<td>FP4</td>
<td>AGAGTTTGATCCTGGTTCAG</td>
<td>Chlamydiae</td>
</tr>
<tr>
<td>RP1</td>
<td>ACGGTTACCTTGTTACGACTT</td>
<td>Enterics (and most eubacteria)</td>
</tr>
<tr>
<td>RP2</td>
<td>ACGGCTACCTTGTTACGACTT</td>
<td>Most eubacteria</td>
</tr>
<tr>
<td>RP3</td>
<td>ACGGATACCTTGTTACGACTT</td>
<td>Fusobacteria (and most eubacteria)</td>
</tr>
</tbody>
</table>

Table 7.1. Summary of primers used for eubacterial 16S rDNA amplification applied in phylogenetic study (Modified from Weisburg et al., 1991)
Rapid detection and identification of microorganisms in food are essential for ensuring food safety and quality, and also important for investigating microbial interactions and persistent mechanisms in the food ecosystems. In this study, we have developed a real-time PCR based system to detect the presence of spoilage foodborne bacteria, *Alicyclobacillus* spp., and a foodborne pathogen, *Listeria monocytogenes*. Both components were tested for detection specificity, sensitivity and applicability in foods. Using the developed primer-and-probe set and following the sample preparation procedure, it was possible to detect less than 100 cells of *Alicyclobacillus* spp. in juice products within 4-7 h, without cross-reactivity with other common foodborne bacteria. This is a significant improvement to current industrial detection approaches. Rapid detection of *L. monocytogenes* was also successfully accomplished in our laboratory using the developed real-time PCR system.

The contribution of a cell surface component, CluA, and conjugation to biofilm development of Gram-positive bacteria, and the correlation between biofilm formation and horizontal gene transfer were illustrated in the foodborne bacterium, *Lactococcus lactis*. Our results showed that CluA played an important role in facilitating biofilm accumulation. Conjugation can be an important avenue for microbes to distribute
beneficial traits, such as biofilm development and antibiotic resistance encoding genes, in the ecosystem. Inherited bacterial high frequency conjugation system, as illustrated in *L. lactis*, could further serve as “facilitator” enhancing the dissemination of antibiotic resistance genes.

While single culture biofilm study is important, investigating biofilm development by multiple strains deserves the attention of scientific community. Our finding demonstrated that primary biofilm forming strains probably play a more significant role than other bacteria in the development of mixed-culture ecosystem. Therefore improving general sanitation is critical in minimizing pathogen persistent in the food processing environment. 16S rRNA gene sequence analysis proved to be effective in identification of microbes in the complicated food ecosystems.

Successful development of methods for microbial detection and identification, and biofilm examination will advance the understanding of microbial persistent mechanisms and potential risks, and will promote the development of effective strategies to combat this major problem affecting food safety and quality. The newly developed rapid detection system can potentially have significant impact on food safety, quality and public health, in addition to financial savings to the industry.
BIBLIOGRAPHY


APPENDIX A.

FOOD COMMENSAL MICROBES AS A POTENTIAL MAJOR AVENUE IN TRANSMITTING ANTIBIOTIC RESISTANCE GENES

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RUNNING HEAD: Food commensals in AR transmission

Key Words:
Foods; commensals; antibiotic resistance; horizontal gene transfer; ecosystem

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A.1 Abstract

The rapid emergence of antibiotic resistant (ART) pathogens is a major threat to public health. While the surfacing of ART foodborne pathogens is alarming, the magnitude of the antibiotic resistance (AR) gene pool in foodborne commensals is yet to be revealed. Incidence of ART commensals in dairy, meat, seafood and produce products was examined in this study. Twelve of the 15 retail cheese samples tested were found to contain Tet\(^r\) microbes ranging from \(10^2\) to \(10^7\) CFU per gram of food. All 15 retail produce samples examined contained Em\(^r\) microbes up to \(10^7\) CFU per gram of product. The presence of large populations of ART bacteria in these foods, particularly in many ready-to-eat, "healthy" food items, indicates that the ART bacteria are abundant in the food chain. AR-encoding genes were detected in ART isolates and the main hosts for these genes in several types of foods were further identified. Particularly, *Streptococcus thermophilus*, a commonly used dairy starter culture, was found to be a major carrier organism for AR genes in cheeses, arguing that it might no longer be suitable for at least certain types of cheese production. AR-encoding plasmids were isolated from several foodborne commensals and transmitted to the oral residential bacterium *Streptococcus mutans* via natural gene transformation under laboratory conditions, suggesting the possible transfer of AR genes from food commensals to human residential bacteria via horizontal gene transfer. Since the ART bacteria from food have the potential to modify the human ecosystems by becoming part of the microbiota, or involved in gene swapping during their transient passage through host oral, upper respiratory and GI systems, routine intake of large numbers of ART bacteria through food consumption may play a
significant role in the existence of an increasingly antibiotic-resistant microbiota in the human ecosystems.

A.2 Introduction

The rapid emergence of resistant pathogens to various antibiotics indicates that the surfacing of resistant pathogens untreatable by antibiotics constitutes a real threat to public health (Levy 1998). To effectively combat this problem, establishing a comprehensive understanding of the major pathways in antibiotic resistance (AR) gene dissemination as well as the key mechanisms in the evolution of antibiotic resistant (ART) bacteria is an urgent need.

While horizontal gene transfer among pathogens in the hospital environment has been recognized as an important avenue for the rapid spread of AR genes among pathogens, evidence showed that additional pathways besides the clinical settings also contributed to the spread of ART bacteria, and that the use of antibiotics in animals can also impact human microbiota (Levy et al, 1976; Smith et al., 2002). Various reports on the isolation of ART pathogens from food animals, retail meat products and farm environment as well as the identification of AR genes in these isolates further support the notion that inappropriate use of antibiotics in agriculture and animal production, whether for therapeutic or subtherapeutic purposes, facilitated the emergence of ART pathogens and that the food chain could be another route in transmitting ART pathogens to humans. However, most food related studies, to date, have examined the AR profiles of a specific group of pathogens, such as E. coli O157:H7 (Schmidt et al., 1998; Kim et al., 1994;), Campylobacter sp. (Gaudreau and Gilbert, 1998; Ruiz et al., 1998; Smith et al., 1999, Ge
et al., 2003), Salmonella enterica serovar Typhimurium (Abrahim et al., 1998; Lee et al., 1994; Fey et al., 2000; Threlfall et al., 2000; Chen et al., 2004), and Listeria monocytogenes (Charpentier et al. 1999; Poyart-Salmeron et al., 1992; Roberts et al., 1996; Abrahim et al., 1998). The studies on commensal bacteria were limited and primarily focused on the opportunistic pathogen enterococci (Klein et al., 1998; Cocconcelli et al., 2003; Johnston and Jaykus, 2004). In most of these cases, a standard laboratory enrichment procedure (http://www.fda.gov/cvm/Documents/AppendicesA-6.pdf) is required in order to detect the presence of the ART bacteria. The difficulty in isolating ART bacteria in such investigations masks the real magnitude of the AR problem associated with the food chain.

The objective of this study is to investigate the distribution spectrum and magnitude of antibiotic ART commensal bacteria and AR gene pool in the US food chain, and to discuss its potential impact on public health. Unlike most previous investigations, this study targeted total food microbiota instead of a particular group of microorganisms or pathogens. Food samples were analyzed without any laboratory enrichment procedures. The goal was to detect bacteria resistant to two commonly used antibiotics, tetracycline (Tet) and erythromycin (Em), within the microflora associated with foods. These two antibiotics are heavily used in animal production, and are still therapeutic options for man (Chopra and Roberts 2001; Roberts 2004; Roberts 2005). The presence of several AR markers including ermB, ermC, tetS/M and tetA was examined in selected food isolates. The tetA gene encodes for the drug efflux protein TetA. The tetS/M, ermB and ermC genes encode proteins that can abolish the function of corresponding antibiotics by non-covalent modification (tetS/M) or methylation (ermB and ermC) of the ribosomes. These
genes have previously been identified in both Gram-positive and Gram-negative microbes, wherein they are associated with various mobile elements (Roberts, 1998). Despite the fact that this current study only screened for a limited number of resistance markers, it certainly revealed the high prevalence of ART commensals and AR genes in various food items. These findings have direct implications on public health, as humans consume these commensal bacteria loaded foods on a daily basis.

A.3 Materials and Methods

A.3.1 Food sample preparation and enumeration of total and ART populations

Food samples were purchased from local grocery stores. All food items analyzed were within the expiration date for consumption. Five grams of each sample were aseptically removed from the product packaging and placed in disposable Ziploc bags containing 10 ml of sterile 0.1% peptone water. Bagged samples were then hand-massaged for 10 minutes. Homogenized samples or rinsing liquids were serially diluted and plated on non-selective Plate Count Agar (PCA, Becton Dickinson and Company, Sparks, MD) for non-selective total microbial counting, and on PCA plates containing 16 μg mL⁻¹ of Tet or 8 μg mL⁻¹ of Em (Fisher Biotech, Fair Lawn, NJ) for assessing Tet and Em resistant population. The levels of antibiotics used in selective agar plates were based on that used to screen for ART enterococci. Serially diluted samples were also plated on Difco Lactobacilli MRS Agar (MRS, Becton Dickinson and Company) plates with the proper antibiotics to recover ART lactic acid bacteria, and on Pseudomonas Selective Agar (PSA, EMD Chemicals Inc., Gibbstown, NJ) with the proper antibiotics for ART
*Pseudomonas* species isolation. Plates were incubated at 32°C or 20°C for up to 48 h at temperatures as specified for each sample. The cell numbers reported were the mean values from duplicates.

**A.3.2 Detection of AR genes by conventional PCR**

Conventional PCR was conducted to detect the presence of AR genes in the ART isolates. Bacterial cells from single colonies were re-suspended in 300 µl sterile dH₂O containing 100 µg of 1:1 mixture of 0.5 µm diameter and 0.1 µm diameter glass beads (Biospec Products, Inc, Bartlesville, OK). The sample mixtures were homogenized using the Mini-Bead-Beater-8 (Biospec Products, Inc, Bartlesville, OK) for 2 min at maximum speed. The resulting cell extracts were placed in a boiling water bath for 10-15 min and 5 µl of the supernatant was used as PCR templates. The PCR primers *tet*A-FP 5’GCTACATCCTGCTTGCTTC3’ and *tet*A-RP 5’CATAGATCGCCGTGAAGAGG3’ were used to amplify the 220 bp *tet*A fragment (Ng et al., 2001), *tet*S-FP 5’CATAGACAGCCGTGACCC3’ and *tet*S-RP 5’ATGTTTTTGGAACGCCAGAG3’ were for the 667 bp *tet*S/M fragment (Ng et al., 2001), *erm*B-FP 5’GGAACAGGTAAAGGGC3’ and *erm*B-RP 5’GGTTTAGGATGAAAGC3’ for the 389 bp *erm*B fragment (this study), and *erm*C FP 5’GCCTATTATTTGCTTAATCGTCAAT3’ and *erm*C RP 5’TCAAAAACAATATAGATAGA3’ for the 640 bp *erm*C fragment (Chung et al., 1999). PCR was conducted using reagents as described previously (Luo et al., 2004) and the amplification conditions included an initial step of 3 minutes at 95°C and 35 cycles of 30 seconds at 95°C (melting), 30 seconds at 55°C (annealing), and 30 seconds at 68°C
(extension), using a thermal cycler (iCycler™, Bio-Rad, Hercules, CA). PCR products with expected sizes were purified using a commercial purification kit (QIAquick®, Qiagen, Valencia, CA) following manufacturer’s instruction. DNA sequences of the 16S rRNA, \textit{erm}C, \textit{tet}A gene fragments and around 50% of the \textit{erm}B and \textit{tet}S/M gene fragments were determined using a DNA analyzer (ABI PRISM® 3700, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio State University. The DNA sequences were compared with published Tet or Em resistance gene sequences deposited in the NCBI database.

\textbf{A.3.3 Identification of ART isolates}

ART isolates containing the resistance genes were identified by PCR amplification of the 16S rRNA gene fragment and sequence analysis following procedures as described previously (Connor et al., 2005). The 1.5 kb 16S rRNA gene fragment of the isolates were amplified using the primer pair 5’AGAGTTTGATCCTGGCTCAG 3’ and 5’TACCTTGTTACGACTT 3’ by PCR (Weisburg et al., 1991), and the sequences of the fragments were determined and compared with those deposited in the NCBI database.

\textbf{A.3.4 MIC profiles of ART isolates}

The minimum inhibition concentration (MIC) profiles of selected ART isolates were determined using the commercial kit Sensititre® 18-24 Hour MIC and Breakpoint Susceptibility Plates (TREK Diagnostic Systems, Cleveland, OH) following the manufacturer’s instructions, with modifications. MRS or brain heart infusion (BHI)
broth instead of the standard Mueller-Hinton broth was used to culture fastidious organisms. The MIC panels were incubated at either 30°C or 37°C for 24-48 h. The MICs were reported as the minimum concentration of the antibiotic that inhibited visible growth, as indicated by increased turbidity or by deposition of cells at the bottom of the wells. Control strains used in the study include *S. aureus* ATCC 29213 [American Type Culture Collection (ATCC), Manassas, VA], *P. aeruginosa* ATCC27853 (ATCC), *L. lactis* ML3 (Kuhl et al., 1979), and *S. thermophilus* LMD-9 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=13773).

A.3.5 Plasmid isolation from selected antibiotic resistant isolates

In this study, the lactococcal strains CZ-T4 (Tet') and CZ-T8 (Tet') were isolated from commercial cheddar cheese, while strain RMK-T14 (Tet') was obtained from raw milk. The multi-drug resistant *L. lactis* K214 was isolated from soft cheese made from raw milk (Perreten et al., 1997). The strains were grown in either MRS broth or M17 broth with 0.5% glucose, supplemented with 5 µg ml⁻¹ Tet and incubated at 30°C for 24 h. Plasmids were isolated from these strains following the method of Anderson and McKay (1983) and were used in the natural transformation experiments.

A.3.6 Natural gene transformation

Overnight cultures of *S. mutans* UA 159 grown in either BHI or Bacto Todd-Hewitt broth (Becton Dickinson and Company) were transferred to fresh medium and incubated at 37°C until the OD₆₀₀ reading reached approximately 0.15-0.30.
Transforming plasmid DNA was then added at a final concentration of 1 µg mL⁻¹. The cultures were allowed to grow for an additional 2 h at 37°C. After the incubation period, the cultures were briefly vortexed and plated on selective and non-selective BHI plates. For the selection of Tet⁺ transformants, BHI plates were supplemented with 5 µg mL⁻¹ Tet. Plates were incubated in a 5% CO₂ incubator at 37°C for 48 h. Transformation efficiency was calculated based on the ratio of Tet⁺ transformants to the total number of viable cells.

A.4 Results

A.4.1 Prevalence of ART bacteria in food samples

Using the screening conditions as indicated, ART bacteria were detected in the majority of the retail food items examined (Table 1), except processed cheese and yogurt (data not shown). High counts of ART microbes were detected not only from raw food materials such as meat and shrimp, but also from many ready-to-eat food items. Twelve out of the 15 cheese samples analyzed contained Tet⁺ microbes ranging from 10² to 10⁷ CFU g⁻¹ of food. The number of Tet⁺ microbes was greater in cheeses than was Em⁺ bacteria. Among 15 produce samples examined (7 reported in Table 1), all contained up to 10⁷ CFU ART microbes per gram of food, and majority of which (20 to 92%) were resistant to Em. The number of Tet⁺ microbes in produce was much lower than that of Em⁺. Since it is unlikely that Em is used in produce production or Tet in cheese fermentation, the reasons for the difference in the magnitudes of Tet and Em-resistant microbes in these ecosystems are unknown. Studies are currently undergoing in our
laboratory to reveal the possible factors contributing to the selective maintenance of these ART bacteria in the corresponding ecological niches.

It is worth of noting that the study was conducted using limited incubation conditions, the antibiotic concentrations used to screen for resistant organisms were based on those used for enterococci and might not be optimal for all bacteria. Therefore the numbers reported here only represent a portion of the total ART bacterial load in these foods.

**A.4.2 Detection of AR genes and ART isolates identification**

To confirm that most of the ART organisms detected by growth on the selective agar plates were resistant bacteria due to the possession of various resistance mechanisms, conventional PCR was conducted to detect the presence of selected AR genes in these organisms. Table 2 summarizes the screening results for some of the Em and Tet markers, and the identities of selected ART isolates as determined by 16S (bacteria) rRNA gene sequence analysis.

Among the Tet\(^r\) isolates recovered from cheese, more than 20% contained the \textit{tet} \textit{S/M} gene (Table 2). The 16S sequence analysis showed that isolates CZ-T4 and CZ-T8 had 97% sequence identity to unidentified \textit{Lactococcus sp.}, and particularly had 93-94% identity to \textit{L. garvieae} and \textit{L. lactis}. Meanwhile, the 16S rRNA sequence analysis of the raw milk ART isolate RMK-T14 showed similar sequence identity to unidentified \textit{Lactococcus sp.} and \textit{L. garvieae}, suggesting this is a common organism from milk. Therefore, it is possible that the cheese lactococcal \textit{tet} \textit{S/M}-containing resistant isolates were originated from milk (pasteurized but not sterile) or dairy processing environment.
during cheese fermentation. *S. thermophilus* was found to be an important carrier for the *tetS/M* gene in cheeses.

Among the Em' isolates from cheese, more than 50% contained the *ermB* gene, and the carrier organisms identified so far include *Staphylococcus* sp. (5 out of 28) and *S. thermophilus* (23 out of 28). Approximately 10-30% of the Em' isolates from salad and carrots contained the *ermB* gene, with the primary carrier organisms being *Pseudomonas* sp. or *Enterobacter* sp. In addition, the *tetA* gene was identified from two cheese isolates CZ-T3, CZ-T7 and several isolates from raw pork meat. These isolates were all identified as *Pseudomonas* sp. Both *tetS/M* and *ermC* genes were found in the isolate CX-I EM from packaged sliced chicken lunchmeat, suggesting a multi-drug resistance phenotype of the strain. CX-I EM was identified to be *Pseudomonas* sp. ART bacteria were isolated sporadically in lunchmeat (data not shown), which is probably due to occasional contamination during the processing of the meat.

**A.4.2 MIC analysis**

MIC tests of selected cheese isolates showed that *Lactococcus* sp. CZ-T4 and CZ-T8 (*tetS/M*') were resistant to at least 16 µg mL$^{-1}$ Tet, and *S. thermophilus* E4 (*ermB*') was resistant to Em (≥ 8 µg mL$^{-1}$), clarithromycin (≥ 8 µg mL$^{-1}$), and clindamycin (4 µg mL$^{-1}$). *Staphylococcus* sp. C202 was resistant to both Em (≥8 µg mL$^{-1}$) and Tet (≥16 µg mL$^{-1}$), suggesting the possible possession of both resistance determinants in this isolate. The control strains *L. lactis* ML3, *S. thermophilus* LMD-9, two more commercial *S. thermophilus* starters and *S. aureus* ATCC 29213 were sensitive to the above antibiotics.
Lactococcus sp. RMK-T14 (tetS/M\(^+\)) from raw milk was resistant to Tet (\(\geq 16 \mu g/mL\)), Em (\(\geq 8 \mu g/mL\)), clarithromycin (\(\geq 8 \mu g/mL\)), and clindamycin (\(\geq 4 \mu g/mL\)). Therefore this isolate likely carried multi-drug resistant determinants or multi-drug resistant mechanism(s). The raw milk isolate Streptococcus uberis RMK-T22W exhibited resistance to Tet (\(\geq 16 \mu g/mL\)).

All of the Pseudomonas tetA\(^+\) isolates recovered from pork and cheese exhibited resistance to Tet (\(\geq 16 \mu g/mL\)), Em (\(\geq 2-8 \mu g/mL\)), and vancomycin (\(\geq 32 \mu g/mL\)), indicating a multi-drug resistance phenotype in these organisms. The Pseudomonas sp. CX-I EM (ermC\(^+\)tetS/M\(^+\)) from packaged sliced chicken lunchmeat was resistant to Tet (\(\geq 16 \mu g/mL\)) and Em (\(\geq 8 \mu g/mL\)).

A.4.3 Horizontal transfer of the AR gene from food isolates to oral residential bacterium

The tetS-containing lactococcal isolates CZ-T4 and CZ-T8, recovered from cheese, and RMK-T14, isolated from raw milk, contained a plasmid with an approximate size of 20-25 kb. To assess the potential risk of the foodborne ART bacteria in disseminating AR genes to human microbiota, plasmids isolated from the above strains were used for natural transformation of the oral cariogenic pathogen *S. mutans* in laboratory media. The tetS/M gene was successfully transferred to *S. mutans* UA159 at frequencies ranging from 1.9 x 10\(^{-7}\) to 2.8 x 10\(^{-5}\), 4.7 x 10\(^{-7}\) to 2.3 x 10\(^{-6}\), and 3.8 x 10\(^{-7}\) to 2.1 x 10\(^{-6}\) transformants per recipient cell using CZ-T4, CZ-T8 and RMK-T14 plasmid extracts, respectively. In addition, the multi-drug resistant plasmid pK214 from the cheese isolate *L. lactis* K214 was also successfully transformed into *S. mutans* UA159 at
frequencies of $1.1 \times 10^{-6}$ to $1.2 \times 10^{-5}$ transformants per recipient cell. PCR amplification confirmed the presence of the $tetS/M$ gene in the streptococcal transformants. MIC test showed that the transformants had significantly increased resistance to Tet ($\geq 16 \mu g mL^{-1}$) compared to the parental strain UA159 (2 $\mu g mL^{-1}$). These results illustrated that the $tetS/M$ gene from food isolates can lead to resistance in residential host bacteria or pathogens, if acquired by horizontal gene transfer.

**A.5 Discussion and Conclusion**

The increasing inability to effectively treat many infections with antibiotics due to a rapidly emerging AR phenotype in many bacteria is a major threat to public health. Effective control strategies to reduce antibiotic resistance need to be built upon having a complete understanding of the key pathways leading to evolution and spread of AR genes as well as identifying the carrier organisms. It has been known that the presence of AR gene pool is the basis for horizontal gene transfer and that selective pressure (antibiotic usage) plays an important role in the enrichment of ART bacteria via horizontal gene transfer (Levy 1998). Horizontal gene transfer among pathogens in the hospital environment is recognized as a main pathway for the rapid spread of AR genes among pathogens, whereas minimizing antibiotic misusage has been the primary control strategy used to combat the AR problem worldwide. The potential impact of antibiotics used in animal production on the emergence of ART pathogens has also been discussed extensively. However, recent studies have showed that the microbiota in children and adults is becoming increasingly resistant to antibiotics, even in the absence of antibiotic
treatment (Lancaster et al., 2003, 2005; Ready et al., 2003; Villedieu et al., 2004). Reports on AR gene reservoirs in the environment and host (Gilliver et al., 1999; Österblad et al., 2001; Nandi et al., 2001; Smith et al., 2004; Salyers et al., 2004), combined with the important new results presented in this study, further indicate that the scope for AR gene transmission is quite broad.

This study targeted the AR gene reservoir in commensal bacteria associated with the food chain. The size of the AR gene pool is quite large in commensals, and horizontal transmissions in ecosystems, directly or indirectly mediated by the abundant and diverse commensal populations, are much more likely events than direct AR gene dissemination from one pathogen to another. Commensal bacteria could even serve as “enhancer” facilitating the dissemination of AR genes in ecosystems (Luo et al, 2005). Our data on the prevalence of ART bacteria can be translated to $10^3$ to $10^8$ CFU Tet$^r$ microbes per slice of cheese (about 20 g), and up to $10^9$ CFU g$^{-1}$ Em$^r$ microbes per serving of salad (about 50-100 g) or baby carrots (about 10 pieces). Since these foods are normally considered healthy, and are consumed without further cooking or processing, these data are a good indication of the daily intake of ART bacteria via the food chain. Therefore without even being exposed to the hospital environment, human beings are unintentionally and constantly inoculated through intake of food with large populations of ART bacteria including opportunistic pathogens and commensals such as Pseudomonas sp., Streptococcus sp. and Staphylococcus sp., many of which carry resistance determinants to antibiotics. This finding is consistent with a previous report that consuming sterile foods can significantly decrease the presence of ART bacteria in the GI system (Levy 1998). Particularly, oral cavity could be an important area where
many initial interactions between food microbes and human microbiota, including horizontal gene transfer events such as conjugation and transformation, took place during the retention of food residues in the oral cavity. In fact, the *tet*S/M and *erm*B genes were found to be abundant in bacteria isolated from foods, which is in agreement with the prevalence of these Tet- and Em-resistance genes in human oral microflora (Roberts, 1998). Successful transmission of the resistance genes from the food isolates to the oral residential bacterium *S. mutans*, by natural gene transformation, further confirmed the functionality of the mobile resistance-encoding elements, if acquired by horizontal gene transfer. Further research is needed to establish the direct correlation between the ART microbes from foods and the ART population in the host ecosystems. However, it is evident that a constant supply of ART bacteria, partnered with occasional colonization and horizontal gene transfer, are at least partially responsible for the increased AR profiles seen in humans.

While the ART population in the human ecosystems might not cause a major problem in healthy people, such an intrinsic AR gene pool could have significant impact on pathogen resistance in susceptible population, and particularly those receiving antibiotic treatment. Due to the magnitude and spectrum of the ART bacteria identified in foods, we propose that in addition to the medical route, the food chain might have served as a major avenue for the transmission of ART bacteria from the environment to human in the general population.

It is worth noting that ART pathogens can emerge in the natural ecosystems and be transmitted to the host, or directly evolve within the host ecosystems. Furthermore, not only are food and related processing environment part of the external ecosystems where
ART bacteria could evolve, but also foods are major vehicles for ART bacteria and selective pressures inherent to the processing procedures themselves (such as addition of preservatives or other food additives), which could further augment the emergence of ART bacteria in the host ecosystems. While it is a major challenge to track the direct and indirect gene transfer events among microbes in complicated ecosystems (Andremont 2003), identifying key AR gene carrier organisms in foods not only reveals the ultimate consequence of these events in the food chain and the organisms involved in horizontal gene transfer, but opens the door for further characterization of conditions in these ecosystems that might facilitate horizontal gene transfer and features of the organisms that might grant their fitness in such ecological niches (Luo et al., 2005a). Such understanding would be critical for effective counteractive strategies to interfere with the detrimental gene swapping in both natural and host ecosystems.

Identification of the key pathways in AR gene transfer is critical but developing a strategy to combat this problem is even more important. Among the foods examined, meat and seafood products are the most subjected to the heat treatment and the ART number would be significantly reduced in cooked foods. Read-to-eat items such as salad and baby carrots are normally consumed raw. Therefore including a bactericidal procedure is important before consumption. Preliminary studies in our group showed that applying minimal heat for short periods (within seconds) or treatments with active ingredients can effectively destroy the ART flora in these products (Lehman et al, unpublished data). Such treatments will not change the sensory feature of the products and will likely lead to manageable approaches for the industry and the consumer to combat the AR problem. The finding of ART bacteria in cheeses often associated with
raw milk, such as *Lactococcus* sp., *Streptococcus* sp. and *Staphylococcus* sp., suggests that cheese fermentation is a susceptible process during which ART bacteria could evolve and proliferate. Improving sanitation and milk heat treatment are thereby an essential step in reducing ART bacteria. Fortunately, traditional starter and adjunct cultures such as *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *Lactobacillus* sp. so far are not among the identified carrier organisms for AR genes. However, an industrially important lactic acid bacterium, *S. thermophilus*, was found a dominant carrier organism for both Tet and Em genes. Genetic screening and MIC tests of three commercial *S. thermophilus* starter cultures showed that the strains are free of these AR genes, suggesting that the susceptibility of this organism to horizontal gene transfer during at least certain cheese fermentation processes and its unsuitability as cheese starter culture.

In general, while it would be a tedious and likely long-term effort to clean up the AR gene pool in the environment, interrupting the transmission of ART bacteria into humans by focusing our efforts on the food chain could be an effective strategy to combat the AR challenge in humans.
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A.6 References


Table A.1. Prevalence of AR microbes in selected food samples

<table>
<thead>
<tr>
<th>Food Item</th>
<th>Sample Source</th>
<th>Total Plate Count (CFU/g food)</th>
<th>Tetr-resistant Count* (CFU/g food)</th>
<th>Em-resistant Count† (CFU/g food)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheddar Cheese 1†</td>
<td>Store I (Brand A)</td>
<td>3.2x10^7±1.6x10^7</td>
<td>1.1x10^6±1.4x10^6</td>
<td>9.0x10^4±1.4x10^4</td>
</tr>
<tr>
<td>Cheddar Cheese 2 †</td>
<td>Store II (Brand B)</td>
<td>2.9x10^7±9.1x10^7</td>
<td>1.2x10^6±6.7x10^7</td>
<td>9.5x10^4±2.4x10^4</td>
</tr>
<tr>
<td>Cheddar Cheese 3 †</td>
<td>Store II (Brand C)</td>
<td>2.5x10^7±3.1x10^7</td>
<td>4.1x10^6±7.0x10^7</td>
<td>4.3x10^4±1.3x10^4</td>
</tr>
<tr>
<td>Cheddar Cheese 4 †</td>
<td>Store II (Brand D)</td>
<td>5.5x10^7±3.5x10^7</td>
<td>1.1x10^6±1.0x10^7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cheddar Cheese 5 †</td>
<td>Store I (Brand E)</td>
<td>1.9x10^7±4.4x10^7</td>
<td>8.0x10^6±3.2x10^7</td>
<td>4.0x10^4±4.0x10^4</td>
</tr>
<tr>
<td>Cheddar Cheese 6 †</td>
<td>Store I (Brand B)</td>
<td>7.6x10^7±2.8x10^7</td>
<td>1.8x10^6±2.0x10^7</td>
<td>2.0x10^4±2.0x10^4</td>
</tr>
<tr>
<td>Cheddar Cheese 7 †</td>
<td>Store III Brand (B)</td>
<td>3.9x10^7±9.1x10^7</td>
<td>3.7x10^6±1.5x10^7</td>
<td>3.7x10^4±7.0x10^4</td>
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<tr>
<td>Cheddar Cheese 8 †</td>
<td>Store I (Brand A)</td>
<td>2.3x10^7±3.6x10^7</td>
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<tr>
<td>Cheddar Cheese 9 †</td>
<td>Store III (Brand C)</td>
<td>5.2x10^7±5.2x10^7</td>
<td>&lt;1</td>
<td>1.0x10^5±1.0x10^5</td>
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<tr>
<td>Colby Cheese #1</td>
<td>Store I (Brand C)</td>
<td>5.6x10^7±4.4x10^7</td>
<td>2.3x10^6±6.1x10^7</td>
<td>6.0x10^4±0.0</td>
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<tr>
<td>Colby Cheese #2</td>
<td>Store II (Brand B)</td>
<td>2.2x10^7±2.8x10^7</td>
<td>2.8x10^6±8.6x10^7</td>
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<td>Store I (Brand E)</td>
<td>2.4x10^7±3.1x10^7</td>
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<tr>
<td>Mozzarella Cheese #1</td>
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<td>5.4x10^6±2.0x10^7</td>
<td>&lt;1</td>
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<td>1.3x10^7±1.7x10^7</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
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<td>&lt;1</td>
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<tr>
<td>Baby Carrots #1</td>
<td>Store I (Brand F)</td>
<td>1.2x10^7±2.5x10^7</td>
<td>-</td>
<td>6.7x10^4±1.0x10^4</td>
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<tr>
<td>Baby Carrots #2</td>
<td>Store II (Brand J)</td>
<td>4.5x10^7±5.4x10^7</td>
<td>1.6x10^6±7.3x10^7</td>
<td>1.2x10^4±1.4x10^4</td>
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<tr>
<td>Baby Carrots #3</td>
<td>Store III (Brand K)</td>
<td>9.0x10^7±6.6x10^7</td>
<td>4.1x10^6±3.5x10^7</td>
<td>4.0x10^5±1.9x10^5</td>
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<tr>
<td>Mushroom #1</td>
<td>Store I (Brand G)</td>
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<td>2.4x10^6±4.2x10^7</td>
<td>8.1x10^4±1.4x10^4</td>
</tr>
<tr>
<td>Mushroom #2</td>
<td>Store I (Brand G)</td>
<td>1.5x10^7±1.1x10^7</td>
<td>5.0x10^6±1.4x10^7</td>
<td>1.0x10^4±5.7x10^6</td>
</tr>
<tr>
<td>Salad #1</td>
<td>Store II (Brand H)</td>
<td>2.4x10^7±8.5x10^7</td>
<td>1.9x10^6±7.1x10^7</td>
<td>2.2x10^4±1.4x10^4</td>
</tr>
<tr>
<td>Salad #2</td>
<td>Store II (Brand H)</td>
<td>6.5x10^7±4.1x10^7</td>
<td>2.8x10^6±4.5x10^7</td>
<td>5.4x10^4±2.2x10^4</td>
</tr>
<tr>
<td>Salad #3</td>
<td>Store I (Brand I)</td>
<td>3.0x10^7±2.8x10^7</td>
<td>4.0x10^6±1.7x10^7</td>
<td>1.5x10^4±5.7x10^6</td>
</tr>
<tr>
<td>Salad #4</td>
<td>Store I (Brand J)</td>
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<td>6.0x10^6±2.8x10^7</td>
<td>1.4x10^4±2.1x10^4</td>
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<tr>
<td>Salad #5</td>
<td>Chain Restaurant A</td>
<td>4.8x10^7±2.5x10^7</td>
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<td>Salad #6</td>
<td>Chain Restaurant A</td>
<td>2.0x10^7±7.1x10^7</td>
<td>4.8x10^6±3.4x10^7</td>
<td>5.2x10^4±3.1x10^4</td>
</tr>
<tr>
<td>Shrimp #1</td>
<td>Store III (Brand C)</td>
<td>1.3x10^7±1.4x10^7</td>
<td>6.9x10^6±1.4x10^7</td>
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<tr>
<td>Shrimp #2</td>
<td>Store III (Brand C)</td>
<td>9.3x10^7±9.9x10^7</td>
<td>3.4x10^6±2.8x10^7</td>
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<tr>
<td>Shrimp #3</td>
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<td>Store IV</td>
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<td>Pork Chop #1</td>
<td>Store II</td>
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<td>5.7x10^6±1.4x10^7</td>
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<td>Pork Chop #2</td>
<td>Store II</td>
<td>6.3x10^7±2.8x10^7</td>
<td>1.0x10^6±1.4x10^7</td>
<td>6.0x10^4±2.8x10^7</td>
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<tr>
<td>Raw Milk #1</td>
<td>Pilot Plant</td>
<td>4.5x10^7±2.5x10^7</td>
<td>3.4x10^6±3.5x10^7</td>
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<tr>
<td>Raw Milk #2</td>
<td>Pilot Plant</td>
<td>8.5x10^7±5.1x10^7</td>
<td>4.8x10^6±2.0x10^7</td>
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<tr>
<td>Raw Milk #3</td>
<td>Pilot Plant</td>
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<td>7.0x10^6±1.2x10^7</td>
<td>-</td>
</tr>
</tbody>
</table>

†Screened on agar plates containing 16µg/ml tetracycline.
‡Screened on agar plates containing 8µg/ml erythromycin.
§Microorganisms were recovered from cheese samples by plating on MRS agar plate and incubated at 30°C.
¶Microorganisms were recovered by plating on PCA agar plate and incubated at 20°C.
⁎Microorganisms were recovered by plating on PCA agar plates and incubated at 37°C.

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<table>
<thead>
<tr>
<th>Food</th>
<th>ART trait</th>
<th>Resistance gene (# carriers /# isolates screened)</th>
<th>16S rRNA gene identity (#organisms/ #identified)</th>
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<tbody>
<tr>
<td>Cheese</td>
<td>Tet</td>
<td>TetS/M (8/33)</td>
<td>Lactococcus sp. (2/8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Streptococcus thermophilus (5/8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>tetA (2/33)</td>
</tr>
<tr>
<td></td>
<td>Em</td>
<td>ermB (32/56)</td>
<td>Staphylococcus sp. (5/28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Streptococcus thermophilus.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(23/28)</td>
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<tr>
<td>Raw milk</td>
<td>Tet</td>
<td>tetS/M (8/108)</td>
<td>Lactococcus sp. (1/8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Streptococcus sp. (1/8)</td>
</tr>
<tr>
<td>Salad</td>
<td>Em</td>
<td>ErmB (7/20)</td>
<td>Enterobacter sp. (2/3)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Pseudomonas sp. (1/3)</td>
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Table A.2. Identification of antibiotic-resistant isolates from food based on 16S rRNA gene sequencing.