

CHARACTERIZATION OF GENETIC DETERMINANTS FOR GROWTH AND  
STRUCTURE OF *Listeria monocytogenes* BIOFILMS

A Thesis

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The Faculty of Graduate Studies

of

Lakehead University

by

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## ABSTRACT

### CHARACTERIZATION OF GENETIC DETERMINANTS FOR GROWTH AND STRUCTURE OF *Listeria monocytogenes* BIOFILMS

Nicholas Choy  
Lakehead University, 2006

Advisor:  
Dr. Heidi Schraft

This thesis investigated wild-type *Listeria monocytogenes* EGD and five of its corresponding mutant strains (M1, M4, M7, M11 and M14) for their ability to form biofilms on glass at 22°C and 37°C, with and without shear force. Cell counts of 24 h-old biofilms ranged from  $10^5$  to  $10^7$  cfu/cm<sup>2</sup>. For all strains investigated, biofilms grown without shear force at 22°C had higher mean biofilm cell counts than at 37°C ( $P < 0.05$ ). Biofilms of wild-type *L. monocytogenes* EGD and its mutant M11 (*clpB* gene deleted) were further analyzed with scanning electron microscopy (SEM). SEM imaging of biofilms grown at 37°C without shear force showed that the wild-type strain EGD produced significantly more biofilm cells than its mutant M11. For biofilms grown at 22°C, both strains displayed characteristic three-dimensional biofilms with cells embedded in what is believed to be extracellular polymeric substances (EPS) when dehydrated with hexamethyldisilazane (HMDS). With the aid of confocal scanning laser microscopy (CSLM), biofilm structures of wild type *Listeria monocytogenes* EGD and its corresponding mutant strains were quantitatively described with PHILIP using the image analysis program. Strains were then characterized with a statistical method known as principal components analysis (PCA). Results from this analysis revealed three strains (M14, M11 and M7) having high thickness and surface-to-biovolume ratio when grown under three conditions (22°C with shear, 37°C with and without shear). A complementation *in trans* was then performed on mutant M11 to ascertain that the phenomenon observed regarding biofilm production and structure on glass, could be attributed to the absence of the *clpB* gene.

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## CHAPTER ONE

### LITERATURE REVIEW

In recent years, *Listeria monocytogenes* has become a major public health concern. Before the 1980's, most problems associated with disease caused by *L. monocytogenes* were related to cattle or sheep. This changed with food related outbreaks in Nova Scotia, Massachusetts, California and Texas. As a result, *L. monocytogenes* is now recognized as an important food-borne pathogen that is able to survive long periods of time under adverse conditions and grow at refrigeration temperatures.

*Listeria monocytogenes* is a pathogen that is widely distributed in the environment such as plants, soil, animal, water, dirt, dust, and silage. Because *L. monocytogenes* may be present in slaughter animals and subsequently in raw meat and poultry as well as other ingredients, it can be continuously introduced into the food processing environment. The pathogen can cross-contaminate food contact surfaces, equipment, floors, drains, standing water and employees. In addition, the pathogen can grow in damp environments, establish a niche and form biofilms in the processing environment that are difficult to eliminate during cleaning and sanitizing. This in turn could lead to serious food safety and quality assurance problems in various food processing environments. Other characteristics of *L. monocytogenes* that make it a formidable pathogen to control will be described in this chapter.

In humans, ingestion of the bacteria may be marked by a flu-like illness or symptoms may be so mild that they go unnoticed. A carrier state may also develop which can lead to death. Death is rare in healthy adults; however, the mortality rate may approximate 30 percent in the immunocompromised, newborns, pregnant women, and the elderly. Given its wide distribution, it is not surprising that during the past 2 decades, there have been several large outbreaks of listeriosis (a kind of meningitis caused by *L. monocytogenes*), all of which associated with commercially supplied foods, especially foods containing milk.

Since then, sporadic cases of listeriosis have also been reported, often in workers in contact with diseased animals (Cain and McCann 1986). As a result of several outbreaks of food-borne listeriosis, along with associated research in North America and in Europe (Wehr 1987), an understanding of this bacterium's mechanisms of pathogenicity has been growing rapidly among food manufacturers and government bodies (Cossart and Mengaud 1989). This chapter reviews the many aspects of *L. monocytogenes* as a food-borne pathogen, including its epidemiology, its incidence in ready-to-eat food products, its occurrence in various natural and food processing environments, and its biofilm forming capabilities and characteristics.

### **1.1. Characteristics of *Listeria monocytogenes***

*Listeria* is a genus representing small, short gram-positive non-sporeforming rods. Belonging to the Clostridium sub-branch, the genus *Listeria* consists of 6 species – *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshieri*, *L. grayi*, and *L. monocytogenes* (Rocourt 1999). Within the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic, as evidenced by their 50% lethal dose (LD<sub>50</sub>) ranging from 1.7 x 10<sup>3</sup> to 9.9 x 10<sup>6</sup> in mice (Audurier *et al.* 1980; Golnazarian *et al.* 1989); and their ability to grow in mouse spleen and liver. *L. monocytogenes* is a human pathogen of high public health concern; *L. ivanovii* is primarily an animal pathogen (Seeliger and Jones 1986).

First recognized as an animal pathogen in 1926 (Murray *et al.* 1926), *L. monocytogenes* has since been recognized as being ubiquitous in the environment, and as the causal agent of diseases in fish, fowl, animals, and humans (Nyfedt 1929). *L. monocytogenes* occasionally causes outbreaks with symptoms usually associated with foodborne illness such as nausea and non-bloody diarrhea. Several foods, including corn, chocolate milk, shrimp, rice salad, and various ready-to-eat meat products have also been reported as vehicles (Schlech 2000).

*L. monocytogenes* is a facultative intracellular pathogen which can invade host tissues by inducing phagocytosis in non-phagocytic cells; a phenomenon known as parasite-directed phagocytosis. This invasion of epithelial cells is a key virulence

mechanism for the bacteria which can cross the intestinal wall and result in a number of different manifestations if it progresses beyond initial infection: *L. monocytogenes* can initially survive intracellularly, spread from cell to cell, multiply in the liver and spleen and can cross endothelial barriers, gaining access to the brain, the placenta or other organs. This in turn leads to meningitis/encephalitis of the central nervous system, neonatal disease, keratoconjunctivitis of the eyes, endocarditis of the heart, and affect other organs as well.

*Listeria monocytogenes* has become a major public health concern due to its widespread occurrence in nature and to its ability to grow at refrigeration temperatures. *L. monocytogenes* is able to initiate growth in the temperature range of 0° to 45°C (George *et al.* 1988; Walder *et al.* 1990; Barbosa *et al.* 1994), and can multiply at a relatively low pH (Gay and Cerf 1997). The pH range for its growth was thought to be 5.6 to 9.6, although recently, investigations indicate that the organism can initiate growth in laboratory media at pH values as low as 4.4 (Lou and Yousef 1999). *L. monocytogenes* also has a tolerance for high salt concentration (up to 30%). The bacterium is not only able to grow in the presence of 10 to 12% sodium chloride, but can grow to high populations in moderate salt concentrations (6.5%) (Shahamat *et al.* 1980). These facts indicate that neither refrigeration nor addition of salt may be sufficient to prevent proliferation of *L. monocytogenes* in contaminated foods.

## 1.2. Listeriosis

*L. monocytogenes* causes several illnesses in humans and animals. Foodborne transmission by improperly processed milk, cheese, meat, and vegetables is the most common source of infection, surviving both high and low temperatures. It is sometimes acquired as a zoonosis and is particularly threatening to those with impaired immune systems.

In immunocompromised persons, food contaminated with *L. monocytogenes* may cause severe invasive disease (Goulet *et al.* 1998; Farber and Peterkin 1991; Pinner *et al.* 1992; Bula *et al.* 1995). Few cases of listeriosis have been reported in previously healthy

persons, and these cases have been attributed to exposure to high infective doses (Kaczmarek and Jones 1989; McLauchlin *et al.* 1990; McLauchlin *et al.* 1991). Although listeriosis is known to be transmitted only through contaminated food, only recently has it begun to be considered a gastrointestinal disease. Some of the episodes reported, have involved gastroenteric symptoms such as diarrhea, nausea, vomiting, and abdominal cramps often accompanied by fever (Ho *et al.* 1986; Schwartz *et al.* 1989). Ingestion of *Listeria* was the suspected cause of two small outbreaks of gastroenteric illness (Riedo *et al.* 1994; Salamina *et al.* 1996). However, only recently has it been demonstrated that foodborne listeriosis can present itself as a gastrointestinal illness with fever (Dalton *et al.* 1997; Heitmann *et al.* 1997; Miettinen *et al.* 1999).

#### 1.2.1. Disease and symptoms

Following ingestion of a *L. monocytogenes* contaminated food; individuals may or may not produce any symptoms. Often which, symptoms appear within 1 to 7 days following ingestion and include mild flu-like symptoms with slight fever, abdominal cramps, and diarrhea. The symptoms usually subside within few days, but the individual still sheds *L. monocytogenes* in the feces for some time (Marth 1988; Lovett 1989; Rocourt 1994).

Symptoms are however, more severe for immunocompromised individuals, elderly, and pregnant women. Initially, symptoms are enteric, with nausea, vomiting, abdominal cramps, and diarrhea with fever and headache. Finally, pathogens spread into the body through the bloodstream and eventually they make their way to vital organs, such as the central nervous system. Symptoms include bacteremia (septicemia), meningitis, encephalitis, endocarditis, and others. *L. monocytogenes* is also one of the few infectious microbes that can cross the placenta and invade organ tissues of the fetus in pregnant women. Those infected, experience at worst a mild flu-like infection, but if the fetus is infected, the infection is systemic and highly fatal. Abscesses develop in the liver, lungs and other fetal organs, and frequently the result is spontaneous abortion or stillbirth. If the baby survives birth, it may be seriously ill with meningitis.

*L. monocytogenes* is notable among the pathogens that cause foodborne infections for the high incidence of fatalities in those who develop systemic infections. Fortunately, few exposed individuals develop the fatal form of the disease. Most of the deaths caused by *L. monocytogenes* involve fetuses, newborns, and immunocompromised adults. For these highly sensitive people, the infective dose is considered to be about 100 to 1000 cells. Although not an especially significant human disease for several decades, listeriosis is now a leading cause of infection in kidney transplant patients and is responsible for many cases of fetal damage.

### **1.3. *L. monocytogenes* in Ready-To-Eat Food Products**

Listeriosis has emerged as a major foodborne disease during the past 2 decades due to the increased consumer demand for convenience food that has a fresh-cooked taste, can be purchased ready-to-eat, refrigerated, or frozen, and requires essentially little cooking before consumption. Data obtained during the past 10 years regarding the sources of outbreaks suggest that some foods are more hazardous vehicles of listeriosis than others. Of these, the highest-risk foods are ready-to-eat foods which are stored under refrigeration for long periods of time and those that are contaminated with *L. monocytogenes* at levels of >100 CFU per g or per ml. This observation was also made during investigations of sporadic listeriosis cases (Jurado *et al.* 1993; Inoue *et al.* 2000).

The increased consumption of many types of ready-to-eat foods that are stored for fairly long periods of time, and the fact that many of these foods are consumed without properly reheating or by microwave heating, has given an edge for *L. monocytogenes* to cause disease. In addition, many changes in handling, preparation, and technological developments used for the production of these ready-to-eat foods may have steps that can contaminate food with the pathogens in low levels, which can subsequently reach a higher level during refrigerated storage prior to consumption. Ready-to-eat foods that have been implicated as a source of infection in listeriosis cases include coleslaw, pasteurized milk, raw milk and dairy products, frankfurters, deli meats, soft cheeses, pate, pork tongue in jelly, and smoked fish.

In recent years, due to high mortality rates, ubiquitous nature, and ability to grow at refrigerated temperatures, federal regulatory agencies around the world have declared a zero-tolerance for *L. monocytogenes* in ready-to-eat foods, similar to that for Salmonella. In Canada, the highest priority is given to those foods that have been known to cause listeriosis and to those that have greater than 10 days of shelf life (Farber 2000).

### 1.3.1. Milk

The occurrence of *L. monocytogenes* in milk products is one that is well-documented showing high prevalence compared with other types of food. In 1985, in California, 142 people who consumed a certain brand of soft cheese developed symptomatic listeriosis. Of these, 93 were perinatal cases and 49 were adult cases. Thirty fetuses or newborn infants died, and 18 adults died. Of the 49 adult cases, 48 occurred in people who were immunocompromised or elderly. The plant in which the cheese was produced was in compliance with safety regulations, and pasteurized milk was used to make the cheese. What appears to have happened is that on some occasions, the pasteurizing equipment could not keep up with the flow of raw milk coming into the plant, and raw milk may have gotten into the final product (Linnan *et al.* 1988; Schuchat *et al.* 1991). Farber and Peterkin (1991) concluded that the overall worldwide incidence of *L. monocytogenes* in raw milk was around 2.2%.

Several investigators have found that cells of *L. monocytogenes* suspended in milk were effectively inactivated under high-temperature-short-time (HTST) pasteurization conditions (71°C for 15 s or equivalent), whereas others contended that the bacteria were not completely inactivated (Bearn and Gerard 1958; Bradshaw *et al.* 1985; Donnelly *et al.* 1987). However, there appears to be a general agreement with the observations of the World Health Organization informal study group, which concluded that “pasteurization is a safe process which reduces the number of *L. monocytogenes* in raw milk to levels that do not pose an appreciable risk to human health” (Anonymous 1998). Therefore, the presence of *L. monocytogenes* in a finished product is likely to result from post-pasteurization contamination from environmental sources in the plant. A study done by



Northolt *et al.* (1988) found that *L. monocytogenes* grows in pasteurized milk, with its numbers increasing 10-fold in 7 days at 4°C; and more rapidly than in raw milk when incubated at 7°C. Recognizing the small margin of safety offered by the HTST process, most raw milk processors have since adopted processes that employ temperatures well above the minimum legal requirements for pasteurized milk. Thus, fluid milk that is contaminated after pasteurization and stored under refrigeration may not only attain very high populations of *L. monocytogenes* after 1 week, but also have its bacterial cells multiplied further due to temperature abuse. This was evidenced by a 1994 outbreak in Illinois, where 45 people became sick after consuming chocolate milk containing a high concentration of *L. monocytogenes* serotype 1/2b ( $8.8 \times 10^8 - 1.2 \times 10^9$  cfu per ml) (Dalton *et al.* 1997).

### 1.3.2. Cheeses

*L. monocytogenes* has the ability to survive the manufacture and ripening of many different cheeses, surviving best in cheeses such as Camembert and worst in products such as cottage cheese. Pearson and Marth (1990) reported that during the ripening of cheese, the numbers of *L. monocytogenes* cells may increase (Camembert), decrease gradually (cheddar or Colby), or decrease rapidly during early ripening and then stabilize (blue cheese). Levels of *L. monocytogenes* as high as  $10^7$  CFU/g have been found in some naturally contaminated cheeses (Farber and Peterkin 1991). With soft cheese, the contamination is localized almost exclusively on the surface of the rind (Ryser and Marth 1987). This phenomenon appears to be due to a pH effect, since a wide pH gradient develops in these cheeses during ripening and *L. monocytogenes* growth has been shown to parallel the increase in pH of cheese during ripening. Because of *L. monocytogenes*' wide pH growth range, consumption of soft cheeses by susceptible persons is a risk factor for sporadic and epidemic listeriosis in North America and Europe (Linnan *et al.* 1988; Bille 1989; Schuchat *et al.* 1991; Schuchat *et al.* 1992; Jacquet *et al.* 1995). In 1985 in California, the first recorded outbreak of listeriosis associated with cheese, involved a certain brand of Mexican-style soft cheese, in which 143 people were affected. It was

later found that inadequate pasteurization of milk that was used to prepare the cheese, along with the mixing of raw with pasteurized milk, resulted in the contaminated cheese (Linnan *et al.* 1988; Schuchat *et al.* 1991). *L. monocytogenes*-contaminated soft cheese was also responsible for a 4-year (1983 to 1987) outbreak of 122 listeriosis cases with 31 deaths in Switzerland (Bille 1989), and 33 cases in France in 1995 (Goulet *et al.* 1995). In 1998, several French investigators concluded that 49% of sporadic listeriosis in that country could be attributed to the consumption of soft cheeses (de Valk *et al.* 1998).

As a result of these outbreaks, food manufacturers and regulatory agencies have raised listeriosis to a higher level of concern by recalling contaminated food products and closing down the manufacturing plants involved. In the United States, the Centers for Disease Control and Prevention recommend that pregnant women avoid the consumption of soft cheeses.

### 1.3.3. Meat and poultry products

What has made *L. monocytogenes* a very significant foodborne pathogen is its capability in colonizing the intestinal tracts of various food production animals, which then act as reservoirs for the maintenance and dissemination of this organism. Most of the observed contamination is on the surface. However, Johnson *et al.* (1990) recently found *L. monocytogenes* in the interior muscle cores of 5 of 110 total samples of beef, pork, and lamb roasts. These organisms were probably present in the muscle at the time of slaughter. Thus, a food animal can contain *L. monocytogenes* in its muscle tissue before slaughter or have its carcass contaminated after slaughter. Farber and Peterkin (1999) reported that eating organ meat (kidney, liver or spleen etc.) may be more hazardous than eating muscle tissue, because *L. monocytogenes* tends to concentrate and multiply in those areas. But regardless of the route of contamination of meat, *L. monocytogenes* attaches strongly to the surface of meat which can result in a biofilm which is difficult to remove or inactivate. Several outbreaks involving contaminated meat have been reported. In the United Kingdom, a contaminated pate was the vehicle of a 300-case outbreak from 1989 to 1990 (McLauchlin *et al.* 1991). In 1992, an outbreak of listeriosis in France from

the consumption of potted pork tongue resulted in 279 cases and 63 deaths (Jacquet *et al.* 1995). In addition, two recent (1998 and 2000) multistate outbreaks of listeriosis occurred in the United States that were linked to contaminated frankfurters and turkey deli meat, respectively (Mead 1999; Centers for Disease Control and Prevention 2000).

Chicken also seems to be heavily contaminated with *L. monocytogenes* showing contamination rates ranging from 12 to 60% (Farber and Peterkin 1991). Bailey *et al.* (1990) recently examined the factors influencing colonization of broiler chickens with *L. monocytogenes*. Although *L. monocytogenes* (orally inoculated) does not colonize chickens as easily as do *Salmonella* or *Campylobacter jejuni*, younger birds were more susceptible to colonization than older birds, and there was a dose-related colonization response. For example, in 1-day old chickens, a challenge of  $10^2$  and  $10^6$  *L. monocytogenes* cells resulted in the colonization of 20% (3 of 15) and 73% (11 of 15) of the infected birds, respectively. It is evident that poultry can become contaminated either environmentally during production or from healthy carrier chickens in the processing plant (Genigeorgis *et al.* 1989; Bailey *et al.* 1990).

The multiplication potential for *L. monocytogenes* in meat and poultry products depends very much on the temperature and the pH of the meat, the type of meat, and the amount of competitive flora present. Glass and Doyle (1989) found that growth of *L. monocytogenes* on meat was highly dependent on product type and pH. The organism tended to grow well on meat products with a pH value near or above 6.0, whereas it grew poorly or not at all on meats near or below pH 5.0. Poultry supported the growth of *L. monocytogenes* better than other meats, and roast beef, summer sausage, and hot dogs supported it the least. For roast beef, summer sausage, and hot dogs the inhibitory factors appeared to be pH, combined pH and water activity ( $a_w$ ), and liquid smoke, respectively (Glass and Doyle 1989). *L. monocytogenes* also grew better at 0°C on vacuum-packaged beef of pH 6.0 than on meat of pH 5.6 (Grau and Vanderlinde. 1988). Another study done by Kaya and Schmidt (1989) showed that growth of *L. monocytogenes* on meat was temperature and background flora dependent. At 7°C or below, *L. monocytogenes* was unable to grow in meat with a low initial background microflora ( $10^5$  CFU/g), whereas at 25°C, no growth of *L. monocytogenes* was observed with a background of  $10^7$  CFU/g or higher. Lactobacilli and not pseudomonads appeared to be the major organisms exerting

an antilisterial effect. *L. monocytogenes*, however, grew well in sterile beef stored at temperatures ranging from 4 to 20°C. Studies by several other groups (Johnson *et al.* 1988; Shelef 1989) have shown that *L. monocytogenes* may be unable to grow on meat stored at 4 or 25°C, but others have shown that the organism is definitely capable of growing on meat (Grau and Vanderlinde 1988; Chung *et al.* 1989; Glass and Doyle 1989; Kaya and Schmidt 1989; Dickson 1990). The organism also appears to be quite capable of survival on meat regardless of treatment. For example, freezing, surface dehydration, and simulated spray chilling do not appear to adversely affect its survival (Kaya and Schmidt 1989; Dickson 1990). Gill and Reichel (1989) found *L. monocytogenes* capable of growth on vacuum-packed meat stored at 0, 2, 5, and 10°C.

In light of the multiplication potential for *L. monocytogenes* in meat and poultry products, and the fact that this organism has been implicated as the source of sporadic and epidemic listeriosis, have spurred recalls in the United States, Canada, and the United Kingdom of meat products containing *L. monocytogenes*. A study conducted in the United States identified consumption of unreheated frankfurters and undercooked chicken as risk factors for sporadic listeriosis (Schwartz *et al.* 1989). In Canada, the incidence of *L. monocytogenes* in domestic ready-to-eat foods has declined from 24% in 1989 to 3% in 1992. However, specimens obtained from the environments of establishments producing *Listeria*-positive foods remained constant at 12% between 1989 and 1992 (Farber and Peterkin 1999). This observation is important because it suggests that once *L. monocytogenes* is established in a food processing plant environment, it may persist in that location for several years even if the products produced at that location are *Listeria*-free for several months.

#### 1.3.4. Seafood

The role of seafood in human listeriosis has only recently been looked into. As a result of increased seafood consumption around the world, public health concerns have increased. Implicated as sources of infections for human listeriosis are shrimp, smoked mussels, and imitation crabmeat. In the United States, *L. monocytogenes* has been

isolated from both domestic and imported, fresh, frozen, and processed seafood products, including crustaceans, molluscan shellfish, and finfish (Jinneman *et al.* 1999). Weagant *et al.* (1988), upon examining 57 samples of frozen seafood products, found 15 samples, including shrimp, crabmeat, lobster tail, fin fish, and surimi-based seafood, to be positive for *L. monocytogenes*. Ashie *et al.* (1996) reported that contamination of seafood can occur after harvest at various stages of processing and during intensive handling.

The presence of *L. monocytogenes* in smoked and lightly processed fish products are often a concern because many of these products are commonly eaten without further heating. In several processing facilities producing cold-smoked salmon, primary sources of *L. monocytogenes* were surface areas of frozen or fresh raw fish coming into the plant. As the processing of fish progressed, the organism was transferred to other processing areas which then became secondary sources of the bacterium (Eklund *et al.* 1995; Heinritz and Johnson 1998). Jemmi (1990) tested 377 samples of smoked and marinated fish and found 47 to be positive for *L. monocytogenes*. Of 100 smoked samples, 24% were positive for the organism. Tropical fish and fish products including dried-salted fish were found to be free of *L. monocytogenes*, although *L. innocua* was found in 3 of 10 fresh, and 5 of 14 frozen samples (Fuchs and Surendran 1989).

Lovett *et al.* (1990) examined the growth of *L. monocytogenes* in shrimp, crabmeat, surimi, and white fish stored at 7°C. *L. monocytogenes* inoculated into samples of these products, which had been sterilized prior to inoculation, increased by about 5 orders of magnitude within 14 days. It has been demonstrated that the organism can also grow in nonsterile products including cooked shrimp, cooked lobster, and cold smoked salmon (Farber 1989).

To date, very little work has been done to examine the growth of *L. monocytogenes* in seafood, as its consumption is still very much less when compared to the consumption of meats and cheeses. In North America, frozen cooked shrimp; canned frozen, fresh, and imitation crab meat; smoked salmon; imitation scallops; frozen canned lobster; and surimi products have been found to be contaminated with *L. monocytogenes*, and have been recalled from the market. Still, case-control studies have not identified this group of foods as a major risk for listeriosis (Rocourt *et al.* 2000).

#### **1.4. *L. monocytogenes* in the Natural Environment**

*Listeria monocytogenes*, a bacterium widely distributed in the environment, is commonly found in soil and water and on plant material, particularly decaying plant material. These environments are regarded as the natural habitat of this organism. The bacterium can survive in decayed vegetation, such as aerobically spoiled silage, for up to two years, and during which time, be a source of infection for listeriosis in farm animals. Contamination of water and soil (possibly via land application of sewage or sewage effluents) has also led to contamination of fruits, vegetables, fish, and shell fish, thus transmitting infection. The high prevalence, ubiquitous nature, and the fact that *Listeria* is able to colonize and survive under adverse environmental conditions makes it very unlikely that the food industry can avoid contact with this bacterium.

##### **1.4.1. Soil and Vegetation**

Because *L. monocytogenes* is primarily a soil microorganism, it may be an important source of contamination in plants and vegetables. Soil fertilized with manure, decaying plant material or even sewage sludge can be reservoirs for transmitting this bacterium to plants and vegetables. Humans and animals can then acquire the bacteria by ingestion. Often, the bacterium moves through the animal and human intestinal tract without causing any symptoms or illness; but may enter a food environment during processing or after processing of these animals. This can pose a serious threat to food industries because if the bacterium is allowed to thrive, it can multiply and cross contaminate food processing equipment, and other foods being processed in its vicinity.

Although many different types of vegetables have been analyzed for the presence of *L. monocytogenes* (Laine and Michard 1988; Sizmur and Walker 1988; Breer and Schopfer 1989; Farber *et al.* 1989; Heisick 1989), only potatoes and radishes appear to be regularly contaminated (Heisick 1989). The organism has also been isolated from shrubs, wild grasses and food plants such as corn, cereals and soya beans (Mitslerlich and Marth 1984).

To date, evidence indicates that soil is not a natural reservoir in which *L. monocytogenes* multiplies. Instead, the widespread presence of *L. monocytogenes* in soil is likely due to contamination by decaying vegetation and fecal material. The soil provides a cool, moist environment and the decaying material provides the nutrients, enabling *L. monocytogenes* to survive (Fenlon 1999). *L. monocytogenes* thrives best in wet soil or mud because of its affinity for moist environments.

#### 1.4.2. Water

*L. monocytogenes* have been found present in aqueous environments such as river waters, lakes and streams (Watkins and Sleath 1981). The bacterium can contaminate water through soil erosion, along with major contributions from industrial sewage and human or animal fecal material (Watkins and Sleath 1981). Once present and given the right temperature, water activity and pH, the bacterium can establish itself in a particular aqueous environment and infect fish, fishery products and humans alike. Although waterborne cases of human listeriosis have not been reported, the bacterium has been isolated from raw fish material and from final fishery products suggesting that these products can be contaminated at any point between harvest and consumption. When *L. monocytogenes* is present in fish from a contaminated aqueous environment, it is usually present in very low numbers.

While several reports indicate that fish and fishery products can be frequently contaminated with *L. monocytogenes*, no major outbreaks associated with these products have been reported. This may be due to inadequate surveillance systems in several countries or because not all factors contributing to both sporadic cases and outbreaks associated with fishery products have been identified. Still, a lower number of cases have been linked to fish associated listeriosis outbreaks when compared to listeriosis outbreaks associated with other foods.

### 1.4.3. Food processing environments

*L. monocytogenes* has been found in food processing industries working with meat, milk and other kinds of foods. This bacterium may enter a food processing environment through incoming food materials and on the shoes and clothing of personnel. Once *L. monocytogenes* is in a food processing environment, it may become established on food contact surfaces and non-food contact surfaces. *L. monocytogenes* is most often detected in moist areas such as floor drains, condensed or stagnant water, floors, residues and processing equipment (Cox *et al.* 1989). Upon cleaning, *L. monocytogenes* may also survive in aerosols and pose a recontamination threat.

*Listeria monocytogenes* has been isolated from both dairy and meat processing plants. Post-processing contamination of dairy products is the most likely route of contamination by *L. monocytogenes*. Sources of *L. monocytogenes* in dairy processing plants include the environment (floors and floor drains, especially in areas in and around coolers or places subject to outside contamination) and raw milk.

In meat and poultry processing plants, the presence of *L. monocytogenes* on carcasses is usually attributed to contamination by fecal matter during slaughter. A high percentage (11 to 52%) of animals are healthy fecal carriers. Up to 45% of pigs harbour *L. monocytogenes* in tonsils, and 24% of cattle have contaminated internal retropharyngeal nodes (Skovgaard and Norrung 1989; Buncie 1991). Humans also contribute to *L. monocytogenes* contamination from improper handling or slaughtering of animals. The bacterium has been recovered from workers' hands, shoes and clothing in slaughterhouses with the highest contaminated areas being cow de-hiding and pig stunning and hoisting. Sources of *L. monocytogenes* in the meat processing environment are also highest with wet locations, such as conveyor belts and floor drains. Regulatory agencies in the U.S. have found that among line samples collected at different phases of production, *L. monocytogenes* 1/2a was found in high frequencies in products following peeling and before packaging (following peeling, the products travelled via conveyor belt prior to packaging). The same serotype was also isolated from cooler floors and from conveyor belts. Analysis of the data obtained suggests that conveyor belts may be the main source of product contamination (Doyle 1984; Wenger *et al.* 1990).



It has been stated that pathogenic bacteria, e.g. *Listeria monocytogenes* and *Salmonella typhimurium*, which attach readily to surfaces (Herald and Zottola, 1988; Helke *et al.*, 1993), may establish in biofilms and detach along with pieces of the biofilm into the product. Attached bacteria and biofilms can thus pose a hygiene risk in food processing because cells in a biofilm have been shown to be significantly more resistant to disinfectants and sanitizers than planktonic cells (Ronner and Wong 1993; Arizcun *et al.* 1997). Because of this, an understanding of how and why *L. monocytogenes* attach and form biofilms is needed so that strategies can be developed to manage and control this pathogen in food industries.

### **1.5. Microbial Biofilms**

Biofilms are defined as matrix-enclosed bacterial populations adhered to a surface or to each other. These bacteria excrete extracellular polymers by which they adhere to surfaces. Enclosed in a matrix of primarily polysaccharide material, noncellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. True biofilms may take days or even weeks to develop and they are not necessarily uniform in time and space.

Basically, microbes can be divided into two groups according to their growth phase: planktonic or sessile (Fletcher 1991). In the sessile phase, microbes are attached to a surface, forming biofilm and functioning as a closely integrated community, whereas in the planktonic stage, they live as individual free floating organisms. Microbes prefer to live as sessile organisms because of protection from anti-microbial agents and disinfectants by the polysaccharide matrix (Genigeorgis 1995). Biofilm-associated organisms also differ from their planktonic/ freely suspended counterparts with respect to the genes that are transcribed and hence their overall physiological state.

Biofilms provide an ideal niche for the exchange of extrachromosomal DNA (plasmids). Conjugation (the mechanism of plasmid transfer) occurs at a greater rate between cells in biofilms than their planktonic counterparts (Ehlers and Bouwer 1999;

Roberts *et al.* 1999; Hausner and Wurtz 1999). Ghigo (2001) suggested that medically relevant strains of bacteria that contain conjugative plasmids develop biofilms more readily than those without. He showed that the F conjugative pilus (encoded by the *tra* operon of the F plasmid) acts as an adhesion factor for both cell-surface and cell-cell interactions, resulting in a three-dimensional biofilm of *Escherichia coli*. Without plasmids, these same microorganisms produce only micro-colonies without any further development. The probable reason for enhanced conjugation is that the biofilm environment provides closer cell-to-cell contact. Since plasmids encode resistance to multiple antimicrobial agents, biofilm association also provides a mechanism for selecting and promoting the spread of, bacterial resistance to harmful agents. In addition to plasmids, other factors aiding increased resistance are: metabolic activity (cells in stationary phase); increased age; and extracellular polymeric substance (EPS) composition (Frank and Koffi 1990; Mattila-sandholm *et al.* 1992; Genigeorgis 1995).

Biofilms can form on a wide variety of surfaces in most ecosystems where nutrients are plentiful. Under suitable conditions, biofilms can also be formed by all types of microorganisms, including spoilage and pathogenic ones. Some of these bacteria also have a higher tendency to form a biofilm than others, the most common being *Pseudomonas*, *Enterobacter*, *Flavobacterium*, *Alcaligenes*, *Staphylococcus* and *Bacillus* (Mattila-sandholm *et al.* 1992; Genigeorgis 1995). Therefore, the fact that parts of the biofilm may dislodge from the surface is of concern in the food processing industry due to the risk of contamination of food products. The risk becomes even more serious because bacteria in biofilm may express an increased resistance to disinfectants or antimicrobial agents (Frank and Koffi 1990).

#### 1.5.1. Biofilm formation

Many microbial species have shown distinct developmental steps in biofilm formation, which include initial attachment to a surface, followed by the formation of microcolonies, and finally, maturation of microcolonies into an EPS-encased mature biofilm.

### 1.5.1-1. Initial attachment

Attachment is a time dependent process. The process is believed to begin when bacteria sense certain environmental parameters/ cues which trigger the transition from planktonic growth to life on a surface (Fletcher and Pringle 1986; Nyvad and Kilian 1990; O'Toole and Kloter 1998). Environmental signals that can influence initial attachment are osmolarity, pH, iron/ nutrient availability, oxygen tension, and temperature (Nyvad and Kilian 1990; Fletcher 1996; O'Toole and Kloter 1998). Although the details of the environmental signals triggering biofilm development may vary from organism to organism, it is clear that environmental parameters have a profound impact on the transition between planktonic and biofilm growth.

Several possible mechanisms by which microbial cells attach and form biofilm on solid surfaces have been suggested. One suggestion is that attachment occurs in two stages. In the first stage, which is reversible, a cell is held to the surface by weak forces (electrostatic and Van der Waals forces). The microorganisms are near, but not in actual contact with the surface. In the second stage, a cell produces complex polysaccharide molecules to attach its outer surface to the surface of a food or equipment, and the process is irreversible. While the microbes are bound irreversibly, they scan the surface to detect whether the nutrient level is sufficient enough to live on (Denyer *et al.* 1993; Zottola 1994). Microorganisms first bind reversibly to the organic matter and then irreversibly with their flagella and fimbriae.

Microbial cells require flagella and pili to initiate early attachment processes (Genevaux *et al.* 1996; Pratt and Kolter 1998). Once microbial cells are in close proximity to a surface, flagellum-mediated motility is required for movement parallel to the surface, which in turn contributes to the spread of bacteria across it. Lipopolysaccharide (LPS), an important component of the bacterial outer membrane, also plays a role in initial surface attachment. Studies have shown that LPS increases a cell's ability to interact with hydrophilic surfaces (Makin and Beveridge 1996). And with time, micro-colonies develop into a mature biofilm that is often associated with the production of EPS. As cells adjust to an immobile life on a surface, they lose their flagella and increase the production of EPS.

Attachment can also introduce some heterogeneity in biofilm structure as microbial cells may adhere in different ways. For example, when more than one species is present, colonization patterns may differ as some microorganisms will join existing biofilms, while others will attach to noncolonized areas where they can start a new biofilm (Genigeorgis 1995). In food processing environments, bacteria have been shown to attach less readily to smooth surfaces (Holah 1992; Mattila-Sandholm and Wirtanen 1992; Wirtanen *et al.* 1996), and biofilms are less likely to develop on well-designed and effectively cleaned equipment (Wirtanen 1995).

#### *1.5.1-2. Attachment genetics*

Although many species-specific behaviors exist that reflect the unique requirement of each microorganism, some general concepts hold true in the formation of most bacterial biofilms. Four gram-negative bacteria subjected to genetic analysis thus far (*E. coli*, *P. aeruginosa*, *P. fluorescens*, and *V. cholerae*), have become model organisms for biofilm research. For each of these organisms, mutants with lesions in genes involved in flagellar-mediated motility were hindered in forming biofilms under certain conditions.

In *E. coli*, flagellar-mediated motility is required for both, approaching and spreading across a surface during initial attachment. Organism-surface interactions were found to require type I pili (essential for the initial attachment event to proceed), and the outer membrane protein Ag43 (directly involved in the interaction of the bacterial cell with a surface) (Danese *et al.* 2000). The proteinaceous cell surface structures known as curli have also been implicated in early attachment events (Vidal *et al.* 1998). In a study done using a non-motile *E. coli* strain to select for mutants that gained the ability to attach to polyvinylchloride (PVC), it was found that an isolated gain-of-function allele (which promotes gene expression) in the outer membrane protein R (*ompR*) increased production in curli. This increased production of curli was shown to be required for initial attachment in the non-motile strain (Vidal *et al.* 1998). Other studies have also shown that the EPS known as colonic acid is required for development of the normal *E. coli* biofilm architecture (Danese *et al.* 2000).

The role of surface structures in *V. cholerae* appears to be similar to that of *E. coli*. Flagella are important for bringing bacteria into close proximity with a surface and for bacterial spread across it. The MshA pili, and possibly one or more unidentified outer membrane proteins, also appear to speed the attachment of bacteria to abiotic surfaces. Studies have shown that *mshA* and flagellar mutants do eventually form biofilms, but at a slower rate than the wild-type (Watnick and Kolter 1999). Initial surface attachment also appears to be stabilized by EPS. In addition, EPS is also needed for formation of wild-type *V. cholerae* mature biofilms and its associated three-dimensional structure.

In addition to flagellar-mediated movement, twitching motility has also been shown to be important for initial biofilm structural development by *P. aeruginosa*. Twitching motility refers to surface-associated movement mediated by type IV pili and appears to be widespread among gram-negative bacteria (Wall and Kaiser 1999). Microscopic analyses of wild-type *P. aeruginosa*, flagellar defective (non-motile) *P. aeruginosa*, and type IV pili defective (twitch negative) *P. aeruginosa* revealed that flagellar-mediated motility is important in bringing bacterium into proximity with the surface, whereas twitching motility appears to play a role in setting up the early structure of the biofilm. Specifically, twitching motility is required for cells to aggregate into microcolonies within the biofilm (O'Toole and Kolter 1998). Studies have revealed that the production of type IV pili is regulated at least in part by nutritional signals via Crc. Crc was shown to regulate *pilA* and *pilB*, encoding the main structural protein of type IV pili and acting as an accessory factor to pilus assembly, respectively (O'Toole *et al.* 2000). LPS was also shown to mediate early interactions, with an additional possible role for outer membrane proteins (*omp*).

LPS, an important component of the bacterial outer membrane, also plays a role in initial surface attachment. Of the two species of LPS produced, the loss of the B-band LPS (but not the A-band LPS) reduced the cell's ability to attach/ interact with hydrophilic surfaces (Makin and Beveridge 1996). Alterations in LPS have also been shown to alter attachment in the related organisms - *P. fluorescens* and *E. coli* (Williams and Fletcher 1996). Interestingly, a genetic analysis of biofilm formation by *P. fluorescens* revealed that it utilizes multiple genetic pathways to initiate biofilm development. For example, some defects in biofilm formation in *P. fluorescens* mutants

conferred by lesions in flagellar genes can be rescued by growth in glutamate, high iron, or citrate (O'Toole and Kolter 1998). This suggests an alternative citrate/ iron-dependent pathway for biofilm formation.

The use of well-characterized mutant strains studied with phase contrast microscopy has proven instrumental in determining the mechanisms by which bacteria can initiate biofilm formation. Although a number of cell surface structures have been shown to be important in early attachment events, their exact role in biofilm development may differ greatly from organism to organism.

### *1.5.1-3. Biofilm structure*

In many cases in the past, biofilms have been handled as if they were compact structures. More recent studies have shown that the biofilm has a porous structure with capillary channels within which water and nutrients are distributed (Costerton *et al.* 1995). The structure/ thickness of a biofilm is dependent on the flow-rate in the surrounding environment, EPS composition and the type and number of different species contained within (Mattila-sandholm and Wirtanen 1992; Costerton *et al.* 1995). Biofilm thickness may also affect the transfer of metabolites into and out of the biofilm, influencing growth rate (Gilbert and Brown 1995; Korber *et al.* 1995).

It can be seen that water channels exist both under and between the microcolonies (Costerton *et al.* 1995). In some cases, these channels in the matrix allow diffusion of nutrients from the vicinity of the biofilm; likewise, they permit the discharge of waste metabolites from the biofilm (Schmidt and Ahring 1996). The transport of nutrients to the bottom and the secretion of wastes are achieved through special capillary tubes facilitated by passive diffusion or water. It is also believed, that the water channels participate in the transport of oxygen to the inner areas of the biofilm. Unfortunately, diffusion limitation (due to the matrix) and oxygen consumption results in only a low oxygen content in the inner biofilm (Genigeorgis 1995; Costerton *et al.* 1995). This is why cells deeply embedded in a biofilm were found to receive less oxygen and fewer nutrients than cells at the surface (Brown *et al.* 1988). This is also the reason why aerobic and anaerobic

microorganisms can be found living together in a biofilm. In addition, cells in the innermost areas of the biofilm were found to be more resistant to antimicrobials and biocides due to decreased amounts/ rates of diffusion. Research has shown that the production of excess amounts of EPS in biofilms may protect the innermost cells by binding with antimicrobial agents/ biocides and detoxifying their effects as they diffuse through the biofilm (Farber *et al.* 1990; Hoyle *et al.* 1990; Stewart 1996; Stewart *et al.* 1998).

#### *1.5.1-4. Attachment to various contact surfaces*

The type of surface material seems to be one crucial factor in biofilm formation. In many technical processes, microbes are not a problem as long as they remain planktonic. In other fields, it would facilitate disinfection if attachment of microbes could be prevented. Several attempts have been made to identify materials that do not promote or even suppress biofilm formation.

The adhesion of bacteria to a surface depends on a number of microbiological, physical, chemical, and material-related parameters. In recent years, surface topography has been widely discussed as a parameter influencing bacterial adhesion. Flint *et al.* (1997) concluded based on review of existing literature that it may be possible to modify surfaces physically or chemically to reduce attachment (e.g. electro-polishing of stainless-steel surfaces) to limit the adhesion of microorganisms. Several parameters or measures have been used to characterize material surfaces, and amongst these, surface roughness (Ra) has been the most widely used. Basically, a small R-factor means that the material is fairly smooth with only has a few bumps or cracks. Glass and polished steel have small R-factors whereas untreated and treated (mechanical grinding, electrolytic polishing) steel have high values. The R-factor can be decreased by further processing. In the food industry, some organizations have set standards for the highest R-factors that are acceptable. For example, in Denmark, the dairies have set a maximum value on surface roughness at 0.8µm when stainless steel is to be used (Friis 1998).

Theoretically, topography of a surface affects the ability of a disinfectant to approach the cell, although recently, it was shown that bacteria are killed/inhibited independent of Ra or topography in general (Boyd *et al.* 2001). Krysiniski *et al.* (1992) showed that stainless steel was more easily cleaned and sanitized than polyester/polyurethane surfaces, despite the observation that no significant topographical differences were detectable (by scanning electron microscopy) among the surfaces to account for this variation in sanitizer resistance. Thus, topography might have an effect on bacterial adhesion/ attachment instead.

In addition to surface roughness and topography, other studies have characterized bacteria and material surfaces with respect to their hydrophobicity and surface free energy using the contact angle method. In general, it is assumed that glass and stainless steel are hydrophilic materials (low contact angles) and rubber and plastic are hydrophobic materials (high contact angles). A study by Sinde and Carballo (2000) revealed that bacteria attached in higher numbers to the more hydrophobic materials than to the hydrophilic ones. In their study, polytetrafluorethylene was the most hydrophobic (and had the lowest surface free energy), followed by rubber and stainless steel. It was also found that the cleaning of materials with commercial sanitizers resulted in a decrease of their contact angles (and an increase in their surface free energies), accompanied by a reduction in the number of adhered bacteria in comparison with the standard conditions (Sinde and Carballo 2000). Polytetrafluorethylene showed the greatest reduction in attachment after being washed with commercial sanitizers as compared to steel and rubber. Sinde and Carballo (2000) thus concluded that stainless steel was less prone to cell adhesion than rubber or polytetrafluorethylene, and should be preferred in the food industry whenever possible. On the other hand, polytetrafluorethylene being more easily sanitized suggested that mixtures of sanitizers be used to help control bacterial adherence in the food industry.

Studies done by other researchers have resulted in similar findings as Sinde and Carballo (2000) with regards to biofilm adhesion to different material surfaces. For example, Rogers *et al.* (1994) ranked different materials according to biofilm growth of microbes in general, and *Legionella pneumophila* in particular. Their results with regard to total microbial count showed that glass and stainless steel were least supportive of



biofilm growth, whereas mild steel, polyethylene and ethylene-propylene were most supportive of biofilm growth. Schoenen and Wehse (1988) examined the microbial count of water samples from pipes and hoses, made from different materials and found that water in contact with PVC and rubber had in general higher counts than water in contact with glass or Teflon. Although the count of the water reflects only planktonic microbes, these results may give indirect hints regarding biofilm formation on various surfaces used in other systems for example, medical or food.

In other studies, attachment to contact surfaces appeared to be species specific. For example, several species and strains of *Pseudomonas* were found to attach to stainless steel surfaces, some within 30 min at 25°C to 2h at 4°C. *Listeria monocytogenes* was found to attach to stainless steel, glass, and rubber surfaces within 20 min of contact (Notermans *et al.* 1991). Another study by Sinde and Carballo (2000) revealed that *L. monocytogenes* strains attached in higher numbers than *Salmonella* strains to steel, rubber and polytetrafluorethylene.

Several attempts have now been made to avoid biofilm formation by incorporation of biocides into surface materials or by coating surfaces with biocides, especially in the medical field, but their significance in biofilm reduction is as yet unknown. On the other hand, thorough cleaning and disinfection of food surfaces can be helpful in reducing biofilms in food processing plants. Many food industries and organizations are now recognizing the importance of microbial attachment and biofilm formation on food contact surfaces. It is apparent from limited studies that this concept is quite wide and needs to be looked into further in order to drastically reduce microbial load in food.

#### *1.5.1-5. Biofilms in food processing environments*

The development of biofilms causes many problems in food industries. Biofilms not only function as reservoirs for diverse species of bacteria, but also provide specific, limited niches and a protective refuge from competitors, predators, or harsh environmental conditions. It is therefore advantageous for microorganisms to adhere to

surfaces, and biofilms to develop if given the right conditions to do so (Korber *et al.* 1995). Microbial adhesion and biofilm formation are a constant concern in food processing environments. Observed and reported on food and food-processing surfaces, bacterial biofilms can contribute to increased risks for product quality and food safety. It is also suspected that biofilms form a unique niche for extended survival of microorganisms in food processing plants (Ronner and Wong 1993; Blackman and Frank 1996; Gravani 1999).

In nature and food systems, microorganisms become attached to solid surfaces conditioned with nutrients, ions and other organic material, in an effort to compete efficiently with other microbial cells for space, nutrient supply and to resist any unfavourable environmental conditions. Microbial adhesion to surfaces is a complex process and studies have shown that bacteria readily adhere to and colonize the surface of any man-made material (Costerton *et al.* 1995). Under favourable conditions, almost all microbial cells are capable of attaching to solid surfaces, which is achieved through their ability to produce extracellular polymeric substances (EPS), usually polysaccharides (Costerton *et al.* 1997). As these cells multiply, they form micro-colonies, giving rise to a biofilm on the surface containing microbial cells, EPS, and entrapped organic material (food debris/soil). In some situations, instead of forming a biofilm, these cells may attach to contact surfaces and other cells by thin, thread-like exopolysaccharide materials, also called fimbriae. It has been shown that exopolymer production by microbial cells is stimulated at a solid surface, and fimbriae have been found to be present on strongly attached cells, but absent from non-attached ones (Cooksey 1992).

During the process of attachment, microbial cells change their phenotype and become basically different from their planktonic counterparts, e.g. express different genes, alter their morphologies and change their growth rates (Gilbert and Brown 1995). Once attached, the exopolymers produced by these cells protect them from adverse environmental conditions (Cooksey 1992). This presents a special problem to the food industry as biofilms can provide some protection to microbial cells against physical removal of the cells by washing and cleaning. These cells also seem to have greater resistance against sanitizers and heat. The presence of viable microorganisms on a

surface presents a 'biotransfer potential' as these microorganisms may detach from the biofilm matrix and contaminate food, causing food-associated diseases or food spoilage.

Pathogenic microorganisms are also known to form or be entrapped in biofilms (Johansen 1997; Holah and Gibson 1999) and once developed, can provide a protective environment for pathogens such as *Listeria monocytogenes* reducing the effectiveness of sanitizers and other inhibitory agents due to increased resistance. Pathogens can thus thrive; and due to the deleterious nature of their film (Zottola and Sasahara 1994), cause biofouling. For example, in many food processing environments, spoilage and pathogenic microorganisms attached to food surfaces, such as carcasses, fish, and meat, cannot be easily removed by washing; and can later multiply and reduce the safety and stability of foods. Similarly, microbial cells attached to equipment surfaces, especially those that come in contact with food, may not be easily killed by chemical sanitizers or heat designed to be effective against unattached microbial cells; and thus can also contaminate food. This problem increases if cleaning and sanitation are delayed following equipment use. Finally, microbial attachment and biofilm formation in the food processing environment, such as floors, walls, and drains, enable the cells to establish in the environment; and they become difficult to control effectively by methods developed and designed against the unattached microorganisms. These places in turn can be a constant source of undesirable microorganisms to foods handled in the environment.

Therefore, it is not only important that microorganisms in the biofilm be killed, but that the remaining polymer matrix be removed as this would provide excellent opportunities for renewed attachment of bacteria and rapid establishment of a new biofilm (Wirtanen 1995). Good sanitation practice is thus recommended to minimize the risk of biofilm formation. Overall, it is important to study the interactions between bacteria and the surfaces in a specific food processing environment to provide more effective measures for prevention and removal of biofilm formation. In addition, a well devised hazard analysis and critical control point (HACCP) plan may also aid in the design of an effective sanitation application.

### 1.5.2. Treatment and removal of biofilms

Removal of microbes and EPS is important in practice to reduce biofilm formation, and the design of effective cleaning and disinfection routines is dependent on expert knowledge and experience. Most often, attached cells are more resistant to biocides than planktonic cells, because the polysaccharide matrix provides a protective barrier limiting the penetration of disinfectants. Thus, to kill and/ or remove biofilm organisms, the biocide used must be able to penetrate the EPS and gain access to the microbial cell. Because the chemical composition of the EPS varies from biofilm to biofilm, nonspecific mechanisms are preferred. Oxidizing substances like chlorine or peroxyacetic acid are frequently used.

In the disinfection of open surfaces, peroxyacetic acid was reported to be superior to chlorine in killing biofilms of *Listeria* and *Pseudomonas* on stainless steel (Fatemi and Frank 1999). Other acid disinfectants composed of hydrogen peroxide and peracetic acid showed the same superiority over chlorine when tested in meat systems. Some researchers have also reported similar results (Carpentier and Cerf 1993; Bourion and Cerf 1996; Fatemi and Frank 1999). For many food industries, thorough cleaning and sanitation done every few hours have become a prerequisite for disinfection.

Hydrolyzing enzymes have also been used as synergists, to boost disinfectant efficacy when administered as an initial treatment along with the use of certain biocides (Jaquelin *et al.* 1994; Johansen *et al.* 1997). In addition, the use of mechanical force applied to a surface during cleaning can significantly influence elimination of biofilms (Gibson *et al.* 1999). Using an automatic scrubber or high pressure cleaning was much more effective in removing biofilm than gel cleaning or low pressure cleaning with disinfection. However, high pressure cleaning may cause more hygiene problems than it solves by spreading surviving microbes via aerosols. Other ways of applying mechanical forces that do not generate aerosols, like slowly running down foam on vertical surfaces or turbulent flow in closed systems, may be preferable. Blanchard *et al.* (1998) demonstrated a 99.7% increase in the efficacy of peroxyacetic acid against biofilms by transition from laminar to turbulent flow in a Robbins device. Thus, any kind of

mechanical action will not only improve cleaning results, but will also result in increased microbial kill, when disinfectants are used.

## 1.6. Objectives of the Study

In most natural and industrial settings, *L. monocytogenes* can often grow attached to surfaces in communities known as biofilms. As reviewed above, biofilm-associated organisms are able to adapt to environmental changes by altering their gene expression and general physiology. This can lead to serious hygiene problems in the food industry as studies have shown that cells within a biofilm are more resistant to sanitizers, antibiotics and other environmental stresses in comparison to their planktonic counterparts. One of the ways in which microbial communities adjust to environmental changes is by changing the structural organization of the biofilm. However, the underlying mechanisms for the initiation of biofilm formation and the development of structural organization are only just beginning to be discovered. By investigating into various *L. monocytogenes* biofilm structures, a greater understanding of biofilm formation can arise which could bring about improvements in sanitization strategies, and ultimately decrease foodborne diseases caused by this pathogen. The objectives of this study were to:

- 1) Compare *L. monocytogenes* mutants for their ability to form biofilms at different treatment levels – 22°C and 37°C, with and without shear force
- 2) Ascertain differences in biofilms produced by *L. monocytogenes* wild-type and mutant strains with scanning electron microscopy (SEM)
- 3) Compare two dehydrating methods commonly used for in preparing biological samples for SEM
- 4) Analyze important biofilm structures of *L. monocytogenes* mutants using confocal scanning laser microscopy (CSLM)
- 5) Characterize important biofilm structures of *L. monocytogenes* mutants with a statistical technique known as Principal Components Analysis (PCA)
- 6) Evaluate the genetic properties of a biofilm defective *L. monocytogenes* mutant of interest by complementation in trans

## References

- Anonymous. 1998. Food listeriosis: Report of the WHO Informal Working Group. WHO/EHE/FOS/88.5. World Health Organization, Geneva, Switzerland.
- Arizcun, C., C. Vasseur, and Labadie, J. C. 1997. Effect of several decontamination procedures on *Listeria monocytogenes* growing in biofilms. *J. Food Prot.* 61:731-734.
- Ashie, I. N. A., J. P. Smith, and Simpson, J. P. 1996. Spoilage and shelf-life extension of fresh and shellfish: Critical reviews in food science and nutrition. 36:87-121.
- Audurier, A., P. Pardon, J. Marly, and Lantier, F. 1980. Experimental infection of mice with *Listeria monocytogenes* and *L. innocua*. *Ann. Microbiol. (Paris)* 131B:47-57.
- Bailey, J. S., D. L. Fletcher, and Cox, N. A. 1990. *Listeria monocytogenes* colonization of broiler chickens. *Poult. Sci.* 69:457-461.
- Barbosa, W. B., L. Cabedo, H. J. Wederquist, J. N. Sofos, and Schmidt, G. R. 1994. Growth variation among species and strains of *Listeria monocytogenes*. *J. Food Prot.* 57:765-769.
- Bearns, R. E. and Gerard, K. F. 1958. The effect of pasteurization on *Listeria monocytogenes*. *Can. J. Microbiol.* 4:55-61.
- Beuchat, L. R., M. E. Berrang, and Brackett, R. E. 1990. Presence and public health implications of *Listeria monocytogenes* on vegetables. In: Miller, A. J., J. L. Smith, and Somkuti, G. A. (eds.), *Foodborne listeriosis*, Elsevier Science Publishing, Inc., New York. pp. 175-181.
- Bille, J. 1989. Presented at "Foodborne Listeriosis," Wiesbaden, Germany, Sept. 7, 1998.
- Blackman, I. C. and Frank, J. F. 1996. Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *J. Food Prot.* 59:827-831.
- Blanchard, A. P., M. R. Bird, and Wright, S. J. L. 1998. Peroxygen disinfection of *Pseudomonas aeruginosa* biofilms on stainless steel disks. *Biofouling* 13(3):233-253.
- Bourion, F. and Cerf, O. 1996. Disinfection efficacy against pure-culture and mixed population biofilms of *Listeria innocua* and *Pseudomonas aeruginosa* on stainless steel, Teflon® and rubber. *Sciences des Aliments* 16:151-166.
- Boyd, R. D., D. Cole, D. Rowe, J. Verran, A. J. Paul, and West, R. H. 2001. Cleanability of soiled stainless steel as studied by atomic force microscopy and time of flight secondary ion mass spectrometry. *J. of Food Prot.* 64:87-93.

- Brown, M. R. W., D. G. Allison, and Gilbert, P. 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J. Antimicrob. Chemother.* 22:777-780.
- Bradshaw, J. G., J. T. Peeler, J. J. Corwin, J. M. Hunt, J. T. Tierney, E. P. Larkin, and Twedt, R. M. 1985. Thermal resistance of *Listeria monocytogenes* in milk. *J. Food Prot.* 48:743-745.
- Breer, C. and Schopfer, K. 1989. *Listerien* in Nahrungsmitteln. *Schweiz. Med. Wochenschr.* 119:306-311.
- Bula, C. J., J. Bille, and Glauser, M. P. 1995. An epidemic of food-borne listeriosis in Western Switzerland: Description of 57 cases involving adults. *Clin. Infect. Dis.* 20:66-72.
- Buncie, S. 1991. The incidence of *Listeria monocytogenes* in slaughtered animals, in meat, and in meat products in Yugoslavia. *Int. J. Food Microbiol.* 12:173-180.
- Cain, D. B. and McCann, V. L. 1986. An unusual case of cutaneous listeriosis. *J. Clin. Microbiol.* 23:976-977.
- Carpentier, B. and Cerf, O. 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Microbiology* 75:499-511.
- Centers for Disease Control and Prevention. 2000. Multistate outbreak of listeriosis: United States. *Morb. Mortal. Wkly. Rep.* 49:1129-1130.
- Chung, K-T., J. S. Dickson, and. Crouse, J. D. 1989. Attachment and proliferation of bacteria on meat. *J. Food. Prot.* 52:173-177.
- Cooksey, K. E. 1992. Extracellular polymers in biofilms. In: Melo L.F., T.R. Bott, M. Fletcher, Capdeville, B. (eds.), *Biofilms: Science and technology*, Kluwer, Dordrecht. pp. 137-147.
- Cossart, P. and Mengaud, J. 1989. *Listeria monocytogenes*: A model system for the molecular study of intracellular parasites. *Mol. Biol. Med.* 6:463-474.
- Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and Lappin-Scott, H. M. 1995. Microbial biofilms. *Annual Reviews of Microbiology.* 49:711-745.
- Costerton, J. W., K-J. Cheng, G. G. Geesey, T. I. Ladd, J. C Nickel, M. Dasgupta, and Marrie, T. J. 1997. Bacterial biofilms in nature and disease. *Annual Reviews of Microbiology.* 41:435-464.
- Cox, L., T. Kleiss, J. Cordier, C. Cordellana, P. Konkel, C. Pedrazzini, R. Beumer, and Siebenga, A. 1989. *Listeria* spp. in food processing, non-food and domestic environments. *Int. J. Food Microbiol.* 6:49-61.

- Dalton, C. B., C. C. Austin, J. Sobel, P. S. Hayes, W. F. Bibb, L. M. Graves, B. Swaminathan, M. E. Procter, and Griffin, P. M. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. *N. Engl. J. Med.* 336:100-105.
- Danese, P. N., L. A. Pratt, S. Dove, and Kolter, R. 2000. The outer membrane protein, Ag43, mediates cell-to-cell interactions in *E. coli* biofilms. *Mol. Microbiol.* 37:424-432.
- Danese, P. N., L. A. Pratt, and Kolter, R. 2000. Exopolysaccharide production is required for development of *E. coli* K-12 biofilm architecture. *J. Bacteriol.* 182:3593-3596.
- de Valk, H., V. Vaillant, V. Pierre, J. Rocourt, C. Jacquet, F. Lequerrec, J. C. Thomas, and Goulet, V. 1998. Risk factors for sporadic listeriosis in France. <http://www.invs.sante.fr/epiet/seminar/1998/valk.html>.
- Denyer, S. P., G. W. Hanlon, and Davies, M. C. 1993. Mechanisms of Microbial Adherence. In: Denyer, S. P., S. P. Gorman and Sussman, M. (eds.), *Microbial biofilms: Formation and control*, Blackwell Scientific Publications, Oxford. pp. 13-27.
- Dickson, J. S. 1990. Survival and growth of *Listeria monocytogenes* on beef tissue surfaces as affected by simulated processing conditions. *J. Food Saf.* 10:165-174.
- Donnelly, C. W., E. H. Briggs, and Donnelly, L. S. 1987. Comparison of heat resistance of *Listeria monocytogenes*. Abstr. 87<sup>th</sup> Annu. Meet. Am. Soc. Microbiol. 1987. American Society for Microbiology, Washington, D. C.
- Doyle, M. P. 1984. Foodborne pathogens of recent concern. *Ann. Rev. Nutr.* 5:1-25.
- Ehlers, L. J. and Bouwer, E. J. 1999. RP4 plasmid transfer among species of *Pseudomonas* in a biofilm reactor. *Water Sci. Technol.* 7:163-171.
- Eklund, M. W., F. T. Poysky, R. N. Paranjpye, L. C. Lashbook, M. E. Peterson, and Pelroy, G. A. 1995. Incidence and sources of *L. monocytogenes* in cold-smoked fishery products and processing plants. *J. Food Prot.* 58:502-508.
- Farber, J. M., M. H. Kaplan, and Colgston, A. G. 1990. *Staphylococcus epidermidis* extracted slime inhibits the antimicrobial action of glycopeptide antibiotics. *J. Infect. Dis.* 161:37-40.
- Farber, J. M., G. W. Sanders, and Johnston, M. A. 1989. A survey of various foods for the presence of *Listeria* species. *J. Food Prot.* 52:456-458.
- Farber, J. M. 1989. Thermal resistance of *Listeria monocytogenes* in foods. *Int. J. Food Microbiol.* 8:285-291.
- Farber, J. M. and Peterkin, P. I. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55(3): 476-511.



- Farber, J. M. and Peterkin, P. I. 1999. Incidence and behavior of *Listeria monocytogenes* in meat products. In: Ryser, E. T. and Marth, E. H. (eds.), *Listeria, Listeriosis, and Food Safety*, 2<sup>nd</sup> ed. Marcel Dekker, Inc., New York. pp. 505-564.
- Farber, J. M. 2000. Present situation in Canada regarding *Listeria monocytogenes* and ready-to-eat seafood products. *Int. J. Food Microbiol.* 62:247-251.
- Fatemi, P. and Frank, J. F. 1999. Inactivation of *Listeria monocytogenes*/*Pseudomonas* biofilms by peracid sanitizers. *Journal of Food Protection.* 62:761-765.
- Fletcher, M. and Pringle, J. H. 1986. Influence of substratum hydration and absorbed macromolecules on bacterial attachment to surfaces. *Appl. Environ. Microbiol.* 51:1321-1325.
- Fenlon, D. R. 1999. *Listeria monocytogenes* in the natural environment. In: *Listeria, Listeriosis, and Food Safety*, 2<sup>nd</sup> ed. Marcel Dekker, New York. pp. 21-38.
- Fletcher, M. 1991. The physiological activity of bacteria attachment to solid surfaces. *Advances in Microbial Physiology.* 32:53-85.
- Fletcher, M. 1996. Bacterial attachment in aquatic environments: A diversity of surfaces and adhesion strategies. In: Fletcher, M. (ed.), *Bacterial adhesion: Molecular and ecological diversity*, John Wiley & Sons, Inc., New York, N.Y. pp. 1-24.
- Flint, S. H., P. J. Bremer, and Brooks, J. D. 1997. Biofilms in dairy manufacturing plant-description, current concerns and methods of control. *Biofouling* 11:81-97.
- Frank, J. F. and Koffi, R. A. 1990. Surface-adherent Growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Protection* 53(7):550-554.
- Friis, A. 1998. Hygiejnisk konstruktion af levnedsmiddelmaskiner. *Mikrobiologi nyt*, 1: 13-16.
- Fuchs, R. S. and Surendran, P. K. 1989. Incidence of *Listeria* in tropical fish and fishery products. *Lett. Appl. Microbiol.* 9:49-51.
- Gay, M. and Cerf, O. 1997. Significance of temperature and preincubation temperature on survival of *Listeria monocytogenes* at pH 4.8. *Lett. Appl. Microbiol.* 25(4):257-260.
- Genevaux, P., S. Muller, and Bauda, P. 1996. A rapid screening procedure to identify mini-Tn10 insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. *FEMS Microbiol. Lett.* 142:27-30
- Genigeorgis, C. A., D. Dutulescu, and Garayzabel, J. F. 1989. Prevalence of *Listeria* spp. in poultry meat at the supermarket and slaughterhouse level. *J. Food Prot.* 52:618-624.

Genigeorgis, C. 1995. Biofilm: Their significance to cleaning in the meat sector. In: Burt, S. A. and Bauer, F. (eds.), *New Challenges in Meat Hygiene: Specific problems in cleaning and disinfection*, Ecceamst, European Consortium for Continuing Education in Advanced Meat Science and Technology. pp. 29-47.

George, S. M., B. M. Lund, and Brocklehurst, T. T. 1998. The effect of pH and temperature on initiation of growth of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* 6:153-156.

Ghigo, J-M. 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature.* 412:442-445.

Gibson, H., J. H. Taylor, K. E. Hall, Holah, J. T. 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology.* 87:41-48.

Gilbert, P. and Brown, M. R. W. 1995. Mechanisms of the protection of bacterial biofilms from antimicrobial agents. In: Lappin-Scott, H.M. and Costerton, J.W. (eds.), *Microbial biofilms*, Cambridge University Press, New York. pp. 118-130.

Gill, C. O. and Reichel, M. P. 1989. Growth of the coldtolerant pathogens *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* on high pH-beef packaged under vacuum or carbon dioxide. *Food Microbiol.* 6:223-230.

Glass, K. A. and Doyle, M. P. 1989. *Listeria monocytogenes* in processed meat products during refrigerated storage. *Appl. Environ. Microbiol.* 55:1565-1569.

Golnazarian, C. A., C. W. Donnelly, S. J. Pintauro, and Howard, D. B. 1989. Comparison of infectious dose of *Listeria monocytogenes* F5817 as determined for normal versus compromised C57B1/6J mice. *J. Food Prot.* 52:696-701.

Goulet, V., J. Rocourt, I. Rebiere, C. Jacquet, C. Moyse, P. Dehaumont, G. Salvat, and Veit, P. 1998. Listeriosis outbreak associated with the consumption of rillettes in France in 1993. *J. Infect. Dis.* 177:155-160.

Goulet, V., C. Jacquet, V. Vaillant, I. Rebiere, E. Mouret, E. Lorente, F. Steiner, and Rocourt, J. 1995. Listeriosis from consumption of raw milk cheese. *Lancet* 345:1581-1582.

Grau, F. H. and Vanderlinde, P. B. 1988. Growth of *Listeria monocytogenes* on vacuum packaged beef. In: *Proceedings of the 34th International Congress of Meat Science and Technology, Part B*. Brisbane, Australia. pp. 518-519.

Gravani, R. 1999. Incidence and control of *Listeria* in food-processing facilities. In: Ryser, E. T. and Marth, E. H. (eds.). *Listeria, Listeriosis, and Food Safety*, 2<sup>nd</sup> edn., Marcel Dekker, New York.

- Hausner, M. and Wuertz, S. 1999. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Appl. Environ. Microbiol.* 65:3710-3713.
- Heinitz, M. L. and Johnson, J. M. 1998. The incidence of *Listeria* spp., *Salmonella* spp., and *Clostridium botulinum* in smoked fish and shellfish. *J. Food Prot.* 61(3):318-323.
- Heisick, J. E., D. E. Wagner, M. L. Nierman, and Peeler, J. T. 1989. *Listeria* spp. Found on fresh market produce. *Appl. Environ. Microbiol.* 55:1925-1927.
- Heitmann, M., P. Gerner-Smidt, and Heltberg, O. 1997. Gastroenteritis caused by *Listeria monocytogenes* in a private day-care facility. *Pediatr. Infect. Dis. J.* 16:827-828.
- Helke, D. M., E. B. Somers, and Wong, A. C. L. 1993. Attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel and Buna-N in the presence of milk and milk components. *J. Food Prot.* 56:479-484.
- Herald, P. J. and Zottola, E. A. 1988. Attachment of *Listeria monocytogenes* to stainless steel surfaces at various temperatures and pH values. *J. Food Prot.* 53:1549-1552, 1562.
- Herald, P. J. and Zottola, E. A. 1989. *J Food Sci* 54: 461-659.
- Ho, J. L., K. N. Shands, G. Friedland, P. Eckind, and Fraser, D. W. 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients in eight Boston hospitals. *Arch. Intern. Med.* 146:520-524.
- Holah, J. T. 1992. Industrial monitoring: hygiene in food processing. In: Melo, L. F., T. R. Bott, M. Fletcher, and Capdeville (eds.) *B.Biofilms – Science and Technology*, Kluwer, Dordrecht. pp. 645-659.
- Holah, J. T. and Gibson, H. 1999. Biofouling: cleanability problems in food processing? In: Wirtanen, G., S. Salo, and Mikkola, A. (eds.). *VTT Symposium 193: 30<sup>th</sup> R<sup>3</sup> – Nordic Contamination Control Symposium*, Helsinki, May 30-June 2. VTT, Espoo, Finland.
- Hoyle, B. D., J. Jass, and Costerton, J. W. 1990. The biofilm glycocalyx as a resistant factor. *J. Antimicrob. Chemother.* 26:1-6.
- Inoue, S., A. Nakama, Y. Arai, Y. Kokubu, T. Maruyama, A. Saito, T. Yoshida, M. Terao, S. Yamamoto, and Kumagai, S. 2000. Prevalence and contamination levels of *Listeria monocytogenes* in retail foods in Japan. *Int. J. Food Microbiol.* 59:73-77.
- Jacquet, C., B. Catimel, R. Brosch, C. Buchrieser, P. Dehaumont, V. Goulet, V. Lepoutre, P. Veit, and Rocourt, J. 1995. Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. *Appl. Environ. Microbiol.* 61:2242-2246.

Jaquelin, L. F., E. Le Magrex, L. Brisset, J. Carquin, A. Berthet, and Choisy, C. 1994. Synergic effect of enzymes or surfactants in association with a phenolic disinfectant on a bacterial biofilm. *Pathologie et Biologie*. 42(5):425-431.

Jemmi, T. 1990. Actual knowledge of *Listeria* in meat and fish products. *Mitt. Geb. Lebensmittelunters. Hyg.* 31:144-157.

Jinneman, K. C., M. M. Wekell, and Eklund, M. W. 1999. Incidence and behavior of *Listeria monocytogenes* in fish and seafood. In: Ryser, E. T. and Marth, E. H. (eds.), *Listeria, Listeriosis, and Food Safety*, 2<sup>nd</sup> ed. Marcel Dekker, Inc., New York. pp. 601-630.

Johansen, C., P. Falholt, and Gram, L. 1997. Enzymatic removal and disinfection of bacterial biofilms. *Applied Environmental Microbiology* 63(9):3724-3728.

Johansen, C. 1997. Enzymes and cationic peptides with antimicrobial activity. Ph. D. Thesis. Institute for Biotechnology, Technical University of Denmark, Lyngby.

Johnson, J. L., M. P. Doyle, and Cassens, R. G. 1988. Survival of *Listeria monocytogenes* in ground beef. *Int. J. Food Microbiol.* 6:243-247.

Johnson, J. L., M. P. Doyle, and Cassens, R. G. 1990. Incidence of *Listeria* spp. in retail meat roasts. *J. Food Sci.* 55:572-574.

Jurado, R., M. Farley, E. Pereira, R. Harvey, A. Schuchat, J. Wenger, and Stephens, D. 1993. Increased risk of meningitis and bacteremia due to *Listeria monocytogenes* in patients with human immunodeficiency virus infection. *Clin. Infect. Dis.* 17:224-227.

Kaczmarek, E. B. and Jones, D. M. 1989. Listeriosis and ready-cooked chicken. *Lancet* 1:549.

Kaya, M. and Schmidt, U. 1989. Verhalten von *Listeria monocytogenes* im Hackfleisch bei Kuhland and gefrierlagerung. *Fleischwirtschaft* 69:617-620.

Korber, D. R., J. R. Lawrence, H. M. Lappin-Scott, and Costerton, J. W. 1995. Growth of microorganisms on surfaces. In: Lappin-Scott, H. M. and Costerton, J. W. (eds.). *Microbial Biofilms*, Cambridge University Press, New York, pp. 15-45

Krysinski, E. P., L. J. Brown and Marchisello, T. J. 1992. Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *J. Food Prot.* 55:246-251.

Laine, K. and Michard, J. 1988. Frequence et abondance des *Listeria* dans des legumes frais d&coups pretsa l'emploi. *Microbiol. Aliments Nutr.* 6:329-335.

- Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, M. L. Yonekura, P. Hayes, R. Weaver, A. Audurier, B. D. Plikaytis, S. L. Fannin, A. Kleks, and Broome, C. V. 1988. Epidemic listeriosis associated with Mexicanstyle cheese. *N. Engl. J. Med.* 319:823-828.
- Lou, Y. and Yousef, A. E. 1999. Characteristics of *Listeria monocytogenes* important to food processors. In: Ryser, E. T. and Marth, E. H. (eds.), *Listeria, Listeriosis, and Food Safety*, 2<sup>nd</sup> ed. Marcel Dekker, Inc., New York. pp. 131-224
- Lovett, J. 1989. *Listeria monocytogenes* in Foodborne Bacterial Pathogens. In Doyle, M. P. (ed.), Marcel Dekker, New York. pp. 283.
- Lovett, J., D. W. Francis, and Bradshaw, J. G. 1990. Outgrowth of *Listeria monocytogenes* in foods. In: Miller, A. J., J. L. Smith, and Somkuti, G. A. (eds.), *Foodborne listeriosis*, Society for Industrial Microbiology, Elsevier Science Publishing, Inc., New York. pp. 183-187.
- Makin, S. A. and Beveridge, T. J. 1996. The influence of A-band and B-band lipopolysaccharide on the surface characteristics and adhesion of *Pseudomonas aeruginosa* to surfaces. *Microbiology* 142:299-307.
- Marth, E. H. 1998. Disease characteristics of *Listeria monocytogenes*. *Food Tech.* 42(2):165-168.
- Mattila-sandholm, T. and Wirtanen, G. 1992. Biofilm formation in the industry: A Review. *Food Reviews International.* 8(4):573-603.
- McLauchlin, J., M. H. Greenwood, and Pini, P. N. 1990. The occurrence of *Listeria monocytogenes* in cheese from a manufacturer associated with a case of listeriosis. *Int. J. Food Microbiol.* 10:255-262.
- McLauchlin, J., S. M. Hall, S. K. Velani, and Gilbert, R. J. 1991. Human listeriosis and pate: A possible association. *Br. Med. J.* 303:773-775.
- Mead, P. S. 1999. Multistate Outbreak of Listeriosis Traced to Processed Meats, August 1998-March 1999. Epidemiologic Investigation Report. Centers for Disease Control and Prevention, Atlanta, Ga.
- Miettinen, M. K., A. Siitonen, P. Heiskanen, H. Haajanen, K. J. Bjorkroth, and Korkeala, H. J. 1999. Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. *J. Clin. Microbiol.* 317:2358-2360.
- Mitscherlich, E. and Marth, E. H. 1984. *Microbial survival in the environment: Bacteria and rickettsia important in human and animal health.* Springer Verlag: Berlin.

- Murray, E. G. D., R. A. Webb, and Swann, M. B. R. 1926. A disease of rabbits characterized by a large monocuclear leucocytosis, caused by a hitherto undescribed bacillus: *Bacterium monocytogenes* (n. sp.). *J. Pathol. Bacteriol.* 29:407-439.
- Northolt, M. D., H. J. Beckers, U. Vecht, L. Toepoel, P. S. S. Soentoro, and Wisselink, H. J. 1988. *Listeria monocytogenes*: Heat resistance and behaviour during storage of milk and whey and making of Dutch types of cheeses. *Neth. Milk Dairy J.* 42:207-219.
- Notermans, S., J. A. M. A. Dormans, and Mead, G. C. 1992. Contribution of surface attachment to the establishment of microorganisms in food processing plants: a review. *Biofouling.* 5:1.
- Nyfedt, A. 1929. Etiologie de la monocucleose infectieuse. *Soc. Biol.* 101:590-592.
- Nyvad, B. and Kilian, M. 1990. Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. *Caries Res.* 24:267-272.
- O'Toole, G. A. and Kolter, R. 1998. The initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. *Mol. Microbiol.* 28:449-461.
- O'Toole, G. A. and Kolter, R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* 30:295-304.
- O'Toole, G. A., K. A. Gibbs, P. W. Hager, P. V. Phibbs Jr., and Kolter, R. 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. *J. Bacteriol.* 182:425-431.
- Pearson, L. J. and Marth, E. H. 1990. *Listeria monocytogenes* – threat to food supply: A review. *J. Dairy Sci.* 73:912-928.
- Pinner, R. W., A. Schuchat, B. Swaminathan, P. Hayes, K. Deaver, R. Weaver, B. Plikaytis, M. Reeves, C. Broome, and Wenger, J. 1992. Role of foods in sporadic listeriosis. 2. Microbiologic and epidemiologic investigation. *JAMA* 287:2046-2050.
- Pratt, L. A. and Kolter, R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: defining the roles of flagella, motility, chemotaxis and type I pili. *Mol. Microbiol.* 30:285-294.
- Riedo, F. X., R. W. Pinner, M. de Lourdes Tosca, M. L. Cartter, L. M. Graves, M. W. Reeves, R. E. Weaver, B. D. Plikaytis, and Broome, C. M. 1994. A point-source foodborne listeriosis outbreak: Documented incubation period and possible mild illness. *J. Infect. Dis.* 170:693-696.

- Roberts, A. P., J. Pratten, M. Wilson, and Mullany, P. 1999. Transfer of a conjugative trasposon, Tn5397 in a model oral biofilm. FEMS Microbial. Lett. 177:636.
- Rocourt, J. 1994. *Listeria monocytogenes*: The state of the science. Dairy Food Environ. Sanit. 14:70.
- Rocourt, J. 1999. The genus *Listeria* and *Listeria monocytogenes*: phylogenetic position, taxonomy, and identification. In: Ryser, E. T. and Marth, E. H. (eds.), *Listeria*, Listeriosis, and Food Safety, 2<sup>nd</sup> ed. Marcel Dekker, Inc., New York. pp. 1-20.
- Rocourt, J., C. Jacquet, and Reilly, A. 2000. Epidemiology of human listeriosis and seafoods. Int. J. Food Microbiol. 62:197-209.
- Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and Keevil, C. W. 1994. Influence of plumbing materials on biofilm formation and growth of *Legionella pneumophila* in potable water systems. Appl. Environ. Microbiol. 60(6):1842-1851.
- Ronner, A. B. and Wong, A. C. L. 1993. Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-N rubber. J. Food Prot. 56:750-758.
- Ryser, E. T. and Marth, E. H. 1987. Fate of *Listeria monocytogenes* during the manufacture and ripening of Camembert cheese. J. Food. Prot. 50:372-378.
- Ryser, E. T. and Marth, E. H. 1987. Behavior of *Listeria monocytogenes* during the manufacture and ripening of cheddar cheese. J. Food Prot. 50:7-13.
- Salamina, G., E. Dalle Donne, A. Niccolini, G. Poda, D. Cesaroni, M. Bucci, R. Fini, M. Maldini, A. Schuchat, B. Swaminathan, W. Bibb, J. Rocourt, N. Binkin, and Salmaso, S. 1996. A foodborne outbreak of gastroenteritis involving *Listeria monocytogenes*. Epidemiol. Infect. 117:429-436.
- Schlech, W. A. 3<sup>rd</sup> 2000. Foodborne listeriosis. Clin. Infect. Dis. 31:770-775.
- Schmidt, J. E. and Ahring, B. K. 1996. Biotechnol. Bioeng. 49:229-246.
- Schoenen, D. and Wehse, A. 1998. Mikrobielle Kontamination des Wassers durch Rohr- und Schlauchmaterialien. 1. Mitt.: Nachweis von Koloniezahlveränderungen. Zentralblatt fuer Bakteriologie und Hygiene B. 186:108-117.
- Schuchat, A., R. W. Pinner, K. Deaver, B. Swaminathan, R. Weaver, P. S. Hayes, M. Reeves, P. Pierce, J. D. Wenger, C. V. Broome, and The Listeria Study Group. 1991. Epidemiology of listeriosis in the USA. In: Amgar, A. (ed.), *Listeria* and Food Safety. ASEPT, Laval, France. pp. 69-73.

- Schuchat, A., C. Lizano, C. V. Broome, B. Swaminathan, C. Kim, and Winn, K. 1991. Outbreak of neonatal listeriosis associated with mineral oil. *Pediatr. Infect. Dis. J.* 10:183-189.
- Schuchat, A., K. Deaver, J. Wenger, B. Plikaytis, L. Mascola, R. W. Pinner, A. Reingold, and Broome, C. 1992. Role of foods in sporadic listeriosis. 1. Case-control study of dietary risk factors. *JAMA* 267:2041-2045.
- Schwartz, B., D. Hexter, C. V. Broome, A. Hightower, R. Hirschorn, J. Porter, P. Hayes, W. Bibb, B. Lorber, and Faris, D. 1989. Investigation of an outbreak of listeriosis: New hypotheses for the etiology of epidemic *Listeria monocytogenes* infections. *J. Infect. Dis.* 159:680-685.
- Seeliger, H. and Jones, D. 1986. *Listeria*. In: Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and Holt, J. G. (eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 2. Williams & Wilkins, Baltimore, Md. pp. 1235-1245.
- Shahamat, M., A. Seaman, and Woodbine, M. 1980. Survival of *Listeria monocytogenes* in high salt concentrations. *Zentbl. Bakteriol. Hyg. Abt. 1 Orig. A* 246:506-511.
- Shelef, L. A. 1989. Survival of *Listeria monocytogenes* in ground beef or liver during storage at 4°C and 25°C. *J. Food Prot.* 52:379-383.
- Sinde, E. and Carballo, J. 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. *J. Food Microbiol.* 17:439-447.
- Sizmur, K. and Walker, C. W. 1988. *Listeria* in prepacked salads. *Lancet* 1:1167.
- Skovgaard, N. and Norrung, B. 1989. The incidence of *Listeria* spp. in faeces of Danish pigs and in minced pork meat. *Int. J. Food Microbiol.* 8:59-63.
- Stewart, P. S. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrob. Agents Chemother.* 40:2517-2522.
- Stewart, P. S., L. Grab and Diemes, J. A. 1998. Analysis of biocide transport limitation in an artificial biofilm system. *J. Appl. Microbiol.* 85:495-500.
- Vidal, O., R. Longin, C. Pringent-Combaret, C. Dorel, M. Hooreman, and Lejune, P. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: Involvement of a new *ompR* allele that increases *curli* expression. *J. Bacteriol.* 180:2442-2449.
- Walder, S. J., P. Archer, and Banks, J. G. 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. *J. Appl. Bacteriol.* 68:157-162.



- Wall, D. and Kaiser, D. 1999. Type IV pili and cell motility. *Mol. Microbiol.* 32:1-10.
- Watkins, J. and Sleath, K. P. 1981. Isolation and enumeration of *Listeria monocytogenes* from sewage, sewage sludge and river water. *J. Appl. Bact.* 50:1-9.
- Watnick, P. I. and Kolter, R. 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol. Microbiol.* In press.
- Weagant, S. D., P. N. Sado, K. G. Colburn, J. D. Torkelson, F. A. Stanley, M. H. Krane, S. C. Shields, and Thayer, C. F. 1988. The incidence of *Listeria* species in frozen seafood products. *J. Food Prot.* 51:655-657.
- Wehr, H. M. 1987. *Listeria monocytogenes*-a current dilemma. *J. Assoc. Off. Anal. Chem.* 70:769-772.
- Wenger, J. D., B. Swaminathan, P. S. Hayes, S. S. Green, M. Pratt, R. W. Pinner, A. Schuchat, and Broome, C. V. 1990. *Listeria monocytogenes* contamination of turkey franks: Evaluation of a product facility. *J. Food Prot.* 53:1015-1019.
- Williams, V. and Fletcher, M. 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. *Appl. Environ. Microbiol.* 62:100-104.
- Wirtanen, G. 1995. Biofilm formation and its elimination from food processing equipment. VTT, Espoo, Finland
- Wirtanen, G., U. Husmark, and Mattila-Sandholm, T. 1996. *J. Food Prot.* 59:727-733.
- Zottola, E. A. 1994. Scientific status, summary, Microbial attachment and biofilm formation, a new problem for the food industry. *Food Tech.* 48(7):107-117.
- Zottola, E. A. and Sasahara, K. C. 1994. Microbial biofilms in the food processing industry: Should they be a concern? *International Journal of Food Microbiology* 23:125-148.

## Chapter Two

### **Biofilm Formation and SEM Analyses of Wild-type and Mutant Strains of *Listeria monocytogenes* on Glass Cover Slips**

#### **2.1. INTRODUCTION**

In most natural and industrial settings, *L. monocytogenes* can grow attached to surfaces, forming communities known as biofilms. Biofilms are highly structured matrix-enclosed microbial communities whose cells express genes in a pattern that differs profoundly from that of their planktonic counterparts (Stoodley et al. 2002). By altering their gene expression and general physiology, biofilm-associated organisms are able to adapt to environmental changes. Often, this leads to serious hygiene problems in the food industry, as studies have shown that cells within a biofilm are more resistant to antibiotics and other environmental stresses.

In recent years, many researchers have incorporated the use of transposon mutants in their studies to gain greater insight into the roles of various genes involved in biofilm formation by pathogenic foodborne bacteria. Biofilm formation is a genetic process that can be affected by various environmental factors such as temperature and shear force. At 22°C, *L. monocytogenes* flagellin genes are expressed aiding in surface attachment, thereby increasing biofilm production, as compared to optimal growth temperature of 37°C (Van Alstyne *et al.* 1969; Way *et al.* 2004). In addition, researchers have shown that shear force significantly influences the structure, mass transfer, production of exopolysaccharides, and metabolic/genetic behaviors of biofilms (Liu and Tay 2002; Tjihuis *et al.* 1996; Gjaltema *et al.* 1994). Reactor studies by Gjaltema *et al.* (1994) showed that even in a well-mixed biofilm reactor (rotatorque reactor) different types of biofilms could be found. These variations arose from slight differences in shear rates at different sites in the reactor. Loosdrecht *et al.* (2002) reported that thinner biofilms were formed with increasing shear rate. This effect has also been observed for other biofilm systems (Verschuren *et al.* 2002).

The morphology of biofilms has not received much attention. For several years, it was assumed that biofilms were homogeneous. With a more detailed analysis of biofilms using scanning electron microscopy (SEM), it is apparent that biofilms are complex structures made up of cell clusters (aggregates of bacterial cells in an exopolysaccharide matrix) and interstitial spaces, contrary to the traditional concept of a planar structure with homogeneous cell distribution. The use of SEM has afforded us a way to not only compare a biofilm defective mutant with its wild-type parent, but also study the effects of temperature on biofilm formation.

Although an excellent tool for visualizing and analyzing biofilms, there has been constant debate over the suitability of two commonly used dehydrating methods for biological sample preparation for SEM analyses – critical point drying (CPD) and drying by hexamethyldisilazane (HMDS). Critical point drying (CPD) is a method used routinely to dry samples for SEM (Boyde and Wood 1969; McCoy *et al.* 1981). However, this technique has several disadvantages, including the time-consuming nature of the process and the complex equipment needed, which increases the cost, especially when there are numerous samples to analyze. Alternatively, air-drying of specimens by the evaporation of HMDS can be used to obtain similar results as those shown for CPD. HMDS is an organic compound, characterized by a good miscibility with standard chemicals used for fixing and preserving biological samples and by a reduced surface tension, which adds strength to the sample during air-drying at room temperature. These characteristics have led to applying HMDS as a pretreatment for air-drying biological samples for SEM study (Botes *et al.* 2002; Dey *et al.* 1989; Nation 1983). Some researchers have even found that drying with HMDS preserves excellent surface detail in insect tissues (Nation 1983) prokaryotic and eukaryotic cells (Dekker *et al.* 1991; Forge *et al.* 1992; Bray *et al.* 1993; Cooley *et al.* 1994). In addition, compared to CPD, drying with HMDS is faster, inexpensive and does not require complex equipment. In spite of the published advantages of HMDS, CPD is still the most frequently used method.

The objectives of this study were to characterize wild-type *Listeria monocytogenes* EGD and five corresponding transposon mutant strains with respect to their planktonic growth at 22°C and 37°C, and their ability to form biofilms on glass under various conditions (22°C and 37°C with and without shear force). In addition, SEM

analysis was used to visualize and compare differences in biofilm production of the biofilm defective mutant M11 and its wild-type parent, and to evaluate differences between two dehydrating methods (HMDS and CPD) used in preparing biological samples for SEM.

## **2.2. MATERIALS AND METHODS**

### **2.2.1. Bacterial Strains**

The pathogenic biofilm-forming *L. monocytogenes* EGD and five transposon insertion mutants were used in this study. The mutants were generated and genetically characterized by Chae (2004). Briefly, transposon Tn916 was inserted into *L. monocytogenes* EGD using a filter mating protocol, and transconjugants were screened for biofilm formation using a microtiter plate quantitative assay. In this test, biofilm-associated cells within the microtiter plate wells were detected by staining with crystal violet. Mutants defective in biofilm formation were thus identified by the absence of crystal violet staining in a well. Important characteristics of the five biofilm defective mutants selected for this study are summarized in Table 2.1.

### **2.2.2. Growth Conditions**

Five biofilm defective mutants of *L. monocytogenes* EGD were used in this study (Table 2.1). For each experiment, the strains were sub-cultured on trypticase soy agar (TSA; Difco Laboratories, Detroit, Michigan, USA) for 24 hours at 37°C with stock cultures prepared weekly and stored at 4°C. For each trial, fresh suspensions were made with isolated colonies and grown in trypticase soy broth (TSB; Difco Laboratories, Detroit, MI) for 24 hours at 37°C. After a 24 hour growth period, the cultures were washed twice by centrifugation (3,100 x g at 4°C for 10 min) in phosphate buffered saline (PBS; 0.13M NaCl, 2.7mM KCl, 5mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). Cell

suspensions were then standardized to  $O.D._{600} = 0.324 \pm 0.007$  using a spectrophotometer. These standardized cell suspensions were then used to grow biofilms as described below. For each standardized suspension, cell densities were recorded and bacterial counts were determined by spiral plating onto TSA incubated for 24 hours at 37°C.

### **2.2.3. Growth Curves**

For each strain, standardized cell suspensions were prepared as described above, enumerated by spiral plate counting ( $\sim 10^8$  CFU/mL) and further diluted to 10 CFU/mL with TSB. Suspensions were incubated at 22°C and 37°C and growth was monitored over 24 hours. At each time interval of 3, 6, 9 and 24 hours, 1mL of the suspension was removed and enumerated by culturing onto TSA with the spiral plater. Colonies were counted after incubating for 24 hours at 37°C. Three replicates were performed for each strain.

### **2.2.4. Growth of Biofilms on Glass Cover Slips**

#### **2.2.4.1. Preparation of glass Petri dishes and cover slips**

Glass cover slips (Fisher premium 24 x 60mm) and glass Petri dishes (100mm diameter) were washed by a 10 minute immersion in 1L of an aqueous 2% RBS Detergent Concentrate solution at 50°C (20mL of RBS 35 Concentrate per liter of tap water; Pierce, Rockford, IL), and then rinsed by immersion in 1L of tap water (initial temp. 50°C) with agitation for 25 minutes. Following that, five additional 1 minute immersions with agitation in 1000mL of distilled water at ambient temperature were performed. Three 2.5cm x 2cm squares were then drawn on each washed glass cover slip with a hydrophobic heat resistant marker (Sharpie industrial permanent marker, Sanford,

Illinois, USA) and allowed to dry. Lastly, each glass cover slip was placed in a washed Petri dish, and the assembly autoclaved at 121°C for 20 minutes.

#### 2.2.4.2. Formation of biofilms

Standardized cell suspensions were prepared for each strain as described above, and 12mL deposited into each sterilized glass Petri dish covering the glass cover slip. The dishes were then closed and placed in larger glass Petri dishes (Pyrex glass petri dish 15cm diameter) containing moist paper towels which provided humidity during incubation at 37°C. After an initial 3 hour incubation period to allow for adhesion to occur, the non-adherent bacteria were removed by slowly pipetting 50mL (25mL x 2) of PBS over the bottom of each of the smaller Petri dishes containing the cover slips. Next, 12mL of TSB was placed over the adherent cells and the dishes were again placed in the larger glass Petri dishes for a 24 hour incubation period at 4 different treatment levels: 22°C and 37°C, with and without shear force. Shear force was achieved by a rotary shaker set at ~ 90 rpm. After 1 day of incubation, the cover slips were rinsed as described above to remove any non-adherent bacteria. The adherent bacteria were then scraped off by moving a cotton swab approximately 100 times over each of the 2.5cm x 2cm squares on each cover glass. Each swab was then placed in a test tube containing 5mL of PBS. The tubes were vigorously vortexed to remove all bacteria from the swabs into the PBS and biofilm bacterial counts were determined by spiral plate counting on TSA. The plates were incubated for 24 hours at 37°C and colonies were enumerated. Each strain was replicated three times for each treatment level.

#### 2.2.5. Scanning Electron Microscopy (SEM) Imaging

Glass cover slips (2.5cm x 2.5cm) were prepared as above and 24 hour biofilms of *L. monocytogenes* EGD and mutant M11 were grown at 22°C and 37°C without shear force. Biofilms were then fixed by immersing them in 2.5% glutaraldehyde at 4°C for 24

hours. Next, the cells were dehydrated by either critical point drying (CPD) or hexamethyldisilazane (HMDS) drying. First, the cover slips were subjected to a graded ethanol series (5%, 10%, 20%, 30%, 50%, 75%, 85%, 95% and 100%). Starting with 5% ethanol, concentrations were increased until 100% was reached. Cover slips were allowed to be immersed for 15 mins at each concentration. The cover slips were then subjected to either CPD or HMDS treatment.

For HMDS drying, the cover slips were transferred from 100% ethanol to a 1:1 ratio of HMDS and 100% ethanol. After 15 mins, the cover slips were placed in a final solution of 100% HMDS and left overnight at room temperature under a fume hood.

After the critical point or HMDS drying step, the cover slips were coated with gold and images for each biofilm were taken at various magnifications (1000x, 3000x and 5000x) using a scanning electron microscope. Two replicates for each strain were analyzed by SEM for each dehydration treatment used.

#### **2.2.6. Statistical Analysis**

One way Anova was used to investigate strain differences between plate count data of 24h biofilms grown with and without shear force at 22°C and 37°C, respectively. The Software package Sigma Stat 2.03 (SPSS Inc.) was used and significance was evaluated at  $P < 0.05$ .

### **2.3. RESULTS**

#### **2.3.1. *L. monocytogenes* Planktonic Cell Growth**

Results for planktonic cell growth of *L. monocytogenes* EGD and its mutants at 22°C and 37°C are shown in Figure 2.1-a,b. For all strains, cell numbers increased continuously from 0 to 24 hours. At 37°C, planktonic growth was similar for all strains. Planktonic cells increased from their initial 10 cfu per mL to  $10^8$  cfu per mL with the

exception of *L. monocytogenes* EGD, which reached only  $10^7$  cfu per mL after 24 hours. However, at 22°C, mutants M11 and M14 showed significantly reduced planktonic growth rates over 24 hours compared to all other strains investigated. Planktonic cells of *L. monocytogenes* mutants M11 and M14 increased slowly from their initial inoculum of 10 cfu per mL to  $10^2$  cfu per mL after 9 hours. At 24 hours, cell counts were  $10^6$  cfu per mL for both mutants M11 and M14. In comparison, all other strains were observed having higher planktonic growth, increasing from 10 to  $10^6$  cfu per mL after 9 hours until finally reaching approximately  $10^8$  cfu per mL after 24 hours.

### 2.3.2. Biofilm Formation on Glass Cover Slips

The initial inoculum of *L. monocytogenes* EGD and five of its corresponding mutant strains was enumerated ( $\text{O.D.}_{600} = 0.324 \pm 0.007$ ) using the spiral plate count method. The average number of cells of all six strains ranged from  $\log_{10}7.83$  cfu/mL to  $\log_{10}8.45$  cfu/mL (Table 2.2).

After subjecting bacterial cells of all strains to 24 hour incubation at four different treatment levels, glass cover slips were found to have attached bacteria ranging from  $10^5$  to  $10^7$  cfu per  $\text{cm}^2$  (Fig. 2.2). Cell counts were generally higher for biofilms grown at 22°C. This difference was more pronounced when shear force was absent, and for this treatment biofilm cell counts of all strains were significantly lower at 37°C than at 22°C ( $P < 0.05$ ).

Mutants grown at 37°C without shear force had lower biofilm cell counts than the wild-type strain, but this difference was only significant for M11. In contrast, when grown at 22°C with and without shear force and at 37°C with shear force, mutants produced similar biofilm cell counts as the wild-type strain. Mutant M7 grown at 37°C with shear force had significantly more biofilm cells than the wild-type strain grown under the same conditions.



### 2.3.3. SEM Analysis of *L. monocytogenes* Biofilms

Results from the SEM analysis of *L. monocytogenes* strain mutant M11 and EGD biofilms grown at 22°C and 37°C without shear force are shown in Figures 2.3-a and 2.3-b respectively. SEM imaging of biofilms grown at 37°C without shear force (Fig. 2.3) confirmed results of plate count analysis (Fig. 2.2), where the wild-type strain EGD produced significantly more biofilm cells (Fig. 2.3-b, Panel K) than its mutant M11 (Fig. 2.3-a, Panel E). When grown without shear force applied, mutant strain M11 also produced significantly lower biofilm cells at 37°C than at 22°C (Fig. 2.2 and Fig. 2.3-a, Panels B and E).

For biofilms grown at 22°C and dehydrated with HMDS, both strains produced characteristic three-dimensional biofilms with cells embedded in what is believed to be extracellular polymeric substances (EPS, Fig. 2.3, Panels A and G). This presumed EPS matrix was not visible when 22°C biofilms were dehydrated with the CPD method (Fig. 2.3, Panel C and I). The matrix was also not seen in biofilms grown at 37°C irrespective of the dehydrating method applied.

## 2.4. DISCUSSION

The time required to reach a particular increase in cell numbers has been used as a means of comparing growth of organisms at different temperatures (Daud *et al.* 1978). As observed in this study, planktonic growth was similar for all strains grown at 37°C for 24 hours. However at 22°C, a reduction in planktonic growth rates of *L. monocytogenes* EGD mutants M11 and M14 was observed. Biofilms of mutant strains M11 and M14 performed almost equally as well as all the other strains studied when grown at 22°C with and without shear force. This suggests that a relationship between planktonic and biofilm cell counts at 22°C after 24 hours did not exist. Therefore, a reduction in planktonic growth rates for mutants M11 and M14 at 22°C could be attributed to a temperature effect on strains possessing loss of function in certain genes. In *L. monocytogenes*, increased expression of mRNAs for *clpB* is induced at low temperature (Liu *et al.* 2002). Thus, a

transposon insertion in the *clpB* gene for mutant M11 might have contributed to the decrease in planktonic growth rates. Mutant M14 on the other hand had a transposon insertion in the *cysK* gene, suggesting the possibility that the *L. monocytogenes cysK* gene was not well expressed in this mutant. Pleog *et al.* (2001) reported that *E. coli* uses the *cysK* gene to encode for cysteine synthase by synthesizing sulphur. Due to the loss of function of this gene in mutant M14, sulphur could not be utilized from nutrients as much as other strains in this study, thus, would explain its decreased growth at 22°C.

*L. monocytogenes* EGD and its five mutants readily developed biofilms on glass cover slips when incubated at 22°C and 37°C with and without shear force applied. Other researchers have also demonstrated the ability of this pathogen to adhere to e.g. stainless steel, polypropylene, and glass slides (Sasahara and Zottola 1993; Ronner and Wong 1993). In this study, temperature and shear force were observed having a significant effect on biofilms cell counts of *L. monocytogenes* wild-type EGD and its mutants. Biofilms grown at 22°C had higher mean biofilm cells counts than at 37°C. A similar finding was also reported by Vantanyoopaisarn *et al.* (2000) who investigated biofilm viable cell numbers of *Listeria monocytogenes* wild-type strain NCTC 7973 (serotype 1/2a), and a nonflagellated (*fla2*) mutant. His studies also suggest that flagella per se facilitate the early stage of attachment and that the difference in attachment of the wild type at 22°C is due primarily to the presence of flagella. At 22°C, the flagellin mutant was found to attach to stainless steel at levels 10-fold lower than wild-type cells, even under conditions preventing active motility. Subsequently at 37°C, when flagella are not produced, attachment of both strains was identical.

Previous studies by Peel *et al.* (1988) and Dons *et al.* (1992) demonstrated that the expression of flagellin in *L. monocytogenes* is temperature dependent – being flagellated and motile at 20 to 25°C. The structural gene for flagellin, *flaA*, was transcribed maximally after growth at 22°C or below, but expression of this gene was repressed at 37°C. Thus, repression of the *flaA* gene may account for the decrease in biofilm cell counts seen at 37°C when shear force was absent. However, with shear force applied, a significant difference between mean biofilm cell counts of all strains grown at 22° and 37°C was not observed. Decreased biofilm cell counts at 22°C with shear force applied, was probably due to detachment caused by particle collision as proposed by Gjaltema *et*

*al.* (1995). A study by Rupp *et al.* (2005) used glass flow cells to demonstrate 'rolling motion'. It suggests that 'rolling motion' is caused by the continual attachment and detachment of the biofilm from a glass surface due to fluid flowing in the flow cell. First, the microcolony detaches from the upstream side, where presumably the fluid shear force overcomes the attachment force of the tether/sticky appendages. It then jerks forward in a rolling motion and the tethers reattach at the downstream side. Our studies indicated that shear force affected cell attachment of all strains similarly, regardless of the temperature applied.

At 37°C without shear, all strains with the exception of mutant M11 performed equally well as its wild-type parent. Mutant strain M11 was observed having significantly lower biofilms cell counts at 37°C compared to its wild-type parent. Even though mutant M11 had a transposon insertion in the *clpB* gene, we cannot attribute this particular loss of gene function to decreased biofilm production at 37°C as this is the optimal growth temperature for *L. monocytogenes* and most other foodborne pathogenic bacteria. The transposon insertion in the *clpB* region which renders that gene inactive is only expressed under stress for e.g. extreme high and low temperatures, high salt or ethanol concentration etc. Thus, one possible reason for mutant M11 having a significantly lower biofilm cell count compared to its wild-type parent would be due to the strain having a lower initial ability to attach to the substratum.

At 37°C with shear, all strains performed equally well, except for mutant M7. Mutant M7 had higher mean biofilm cells counts compared to its wild-type parent. Possible reasons for this include that this strain experienced a decreased detachment effect by shear forces and/or had a higher initial ability to attach to the substratum. It could also be said that the inactive *yfhL* gene in mutant M7 did not affect its biofilm forming capabilities. Mutant M7 had a transposon insertion within the *lmo2435* gene, which was found to be similar to the *B. subtilis* YfhL protein. Thus far, the function of this protein is as yet unknown and further studies are still required to elucidate the actual role of this gene in biofilm production.

Detachment of clumps of pathogenic bacteria from biofilms, or the flow of biofilms across surfaces due to shear forces may be an important consideration in the dissemination of infection to a host from infected food and food contact surfaces. It has

even been proposed that given enough time (days), bacteria in flow systems will attach more strongly to their substratum because of the constant shear force of nutrient medium they have to overcome (Donlan 1992; Donlan and Costerton 2002). Thus, longer incubation times are needed to fully assess the attachment properties of biofilm cells in response to fluid shear stress. It can be concluded that the knowledge regarding the effects of shear force on biofilms is far from complete and much research is still needed to fully understand the relevant mechanisms. Additionally, this information may be useful in designing novel strategies for biofilm removal or stabilization.

The use of SEM afforded us a way to not only ascertain differences in biofilms produced by mutant strain M11 at 22°C and 37°C in comparison to the wild-type EGD strain, but to also compare two commonly used methods for dehydrating biological samples. In this study, all glass cover slips analyzed had attached biofilms. Some biofilms were thick with cells embedded in EPS, while others were thin displaying only single cells.

From images obtained with SEM, biofilms grown at 37°C (regardless of dehydration treatment method applied) exhibited a two-dimensional single layer of cells. In contrast, biofilms grown at 22°C had EPS encased cells (CPD treatment) and three-dimensional cell clusters (HMDS treatment). After attachment to a surface, bacteria adopt new modes of growth, termed biofilm-associated growth. Two properties are often reported for surface-attached bacteria: increased synthesis of EPS and the development of antibiotic resistance. Biofilm formation is a slow process and a mature biofilm only reaches a few micrometers in thickness over a period of several days, depending on the organism and culture conditions (Carpentier and Cerf 1993). At 22°C, increased surface attachment of *L. monocytogenes* wild-type and mutant M11 cells was probably due to the flagellin gene being expressed. These cells were possibly able to increase production of EPS and mature faster compared to those grown at 37°C, as seen after drying with HMDS. Other researchers have also reported that a minimum time of 48 hours was required before *L. monocytogenes* produced a detectable glycocalyx, or EPS, on various surfaces at 37°C (Wirtanen and Mattila-Sandholm 1992).

Our studies also showed that CPD and drying by HMDS produced different SEM images. Drying by HMDS preserved the EPS better than critical point drying for *L.*

*monocytogenes* biofilms grown at 22°C without shear force. Instead, CPD caused complete disappearance of the matrix of extracellular polymeric substances (EPS). A study by Cortizo and de Mele (2003) reported that a reduction of up to half of the original thickness of a biofilm can occur due to dehydration of EPS during CPD. This suggests that SEM pretreatment with CPD can in fact remove biological material or impede the visualization of certain microstructural characteristics.

The surface tension forces generated during evaporation of HMDS did not seem to have any deleterious effects on the biofilms studied. As observed, cell envelopes remained intact and cells appeared to be of similar size as those seen by CPD at the same magnifications. This is in accordance with the findings of Braet *et al.* (1997), Bray *et al.* (1993), and Nation (1983), during their studies with eukaryotic and prokaryotic cells. Thus, drying by HMDS may be more suitable in not only demonstrating EPS by SEM imaging, but preserving the integrity of the EPS matrix and cell envelopes without any apparent shrinkage artefacts. Drying by HMDS appears to be a valuable, time saving and inexpensive alternative to CPD.

Our findings have provided insight into understanding the characteristics, adhesion and formation of *L. monocytogenes* biofilms on glass. Although Chae (2004) found significantly reduced biofilm formation in all *L. monocytogenes* mutants compared to their wild-type parent through his microtiter plate assay, results from our studies do not reflect this. This significant reduction was probably due to different adhesion properties of the mutants with respect to polystyrene and glass. Thus, further studies have to be done to re-evaluate the true situation of *L. monocytogenes* wild-type EGD and its mutants with regard to biofilm growth on various materials.

Our studies have shown that cells of pathogenic *L. monocytogenes* EGD and its mutants can proliferate and spread over a surface forming a biofilm in just 24 hours. This can pose a large unrecognized threat to food industries as biofilms are more resistant to antimicrobial agents, sanitizers and environmental stresses (Costerton *et al.* 1995; Stewart *et al.* 2000). Therefore, a further understanding of the processes involved in biofilm development is needed to guarantee effective countermeasures to help reduce biofilm formation.

**Table 2.1. List of *Listeria monocytogenes* mutant strains used in the study**

<b>Properties of Insert</b>					
<b>Mutant</b>	<b>% of level of wild-type adhesion (a)</b>	<b>Sizes (bp) (b)</b>	<b>% homology with <i>L. monocytogenes</i> EGD (c)</b>	<b><i>L. monocytogenes</i> EGD gene (d)</b>	<b>Function or closest homologue (e)</b>
M1	8.89	123	98	<i>lmo1825 (dfp)</i>	Pantothenate metabolism flavoprotein of <i>L. monocytogenes</i> (AF104226)
M4	8.13	528	98	<i>lmo6091&amp;6092(che YA operon)</i>	Chemotaxis of <i>L. monocytogenes</i> (X76170.1)
M7	14.09	212	98	<i>lmo2435</i>	<i>B. subtilis</i> YfhL protein (CAD00513)
M11	4.44	484	99	<i>lmo2206 (clpB)</i>	Endopeptidase Clp ATP-binding chain B (CAD00284)
M14	9.64	469	100	<i>lmo0223 (cysK)</i>	Cystein synthase (CAD00750)

***Mutant strains were created and characterized by Min Seok Chae, Department of Food Science, University of Guelph (Chae, 2004).***

(a) Wild-type adhesion was considered to be 100%

(b) DNA flanking regions of mutants were blasted against the *L. monocytogenes* EGD database.

(c)-(d) Each sequence of mutants was matched with DNA sequences of *L. monocytogenes* EGD in GenBank by using the BLAST service of the National Center for Biotechnology Information to obtain % DNA homologies.

(e) The function of the protein encoded is listed if it has been supported by genetic or biochemical data. Otherwise, the nearest homologue of the protein with known function is listed. The accession numbers in GenBank database are shown in brackets.

**Table 2.2. Ave. cell numbers and st. dev. of standardized *Listeria monocytogenes* wild-type and mutant cultures used in the growth of biofilms**

Strain	N	Cell numbers (Log CFU/mL)	
		Average	St. Dev.
EGD	24	8.45	0.11
M1	12	8.32	0.082
M4	12	8.30	0.14
M7	12	8.30	0.074
M11	12	7.88	0.13
M14	12	7.83	0.14

- Cell cultures were standardized to  $O.D._{600} = 0.324 \pm 0.007$
- N denotes the number of standardized cell cultures used in this study
- Cell counts were derived by spiral plating

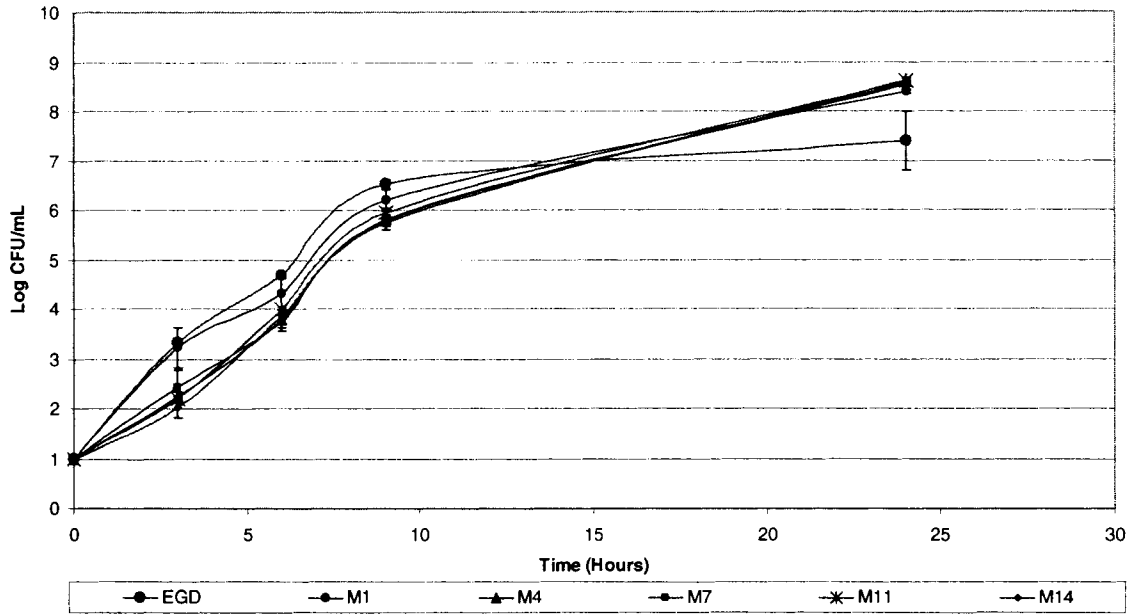


Fig. 2.1-a. Growth curves for *Listeria monocytogenes* wild-type EGD and its mutants at 37°C

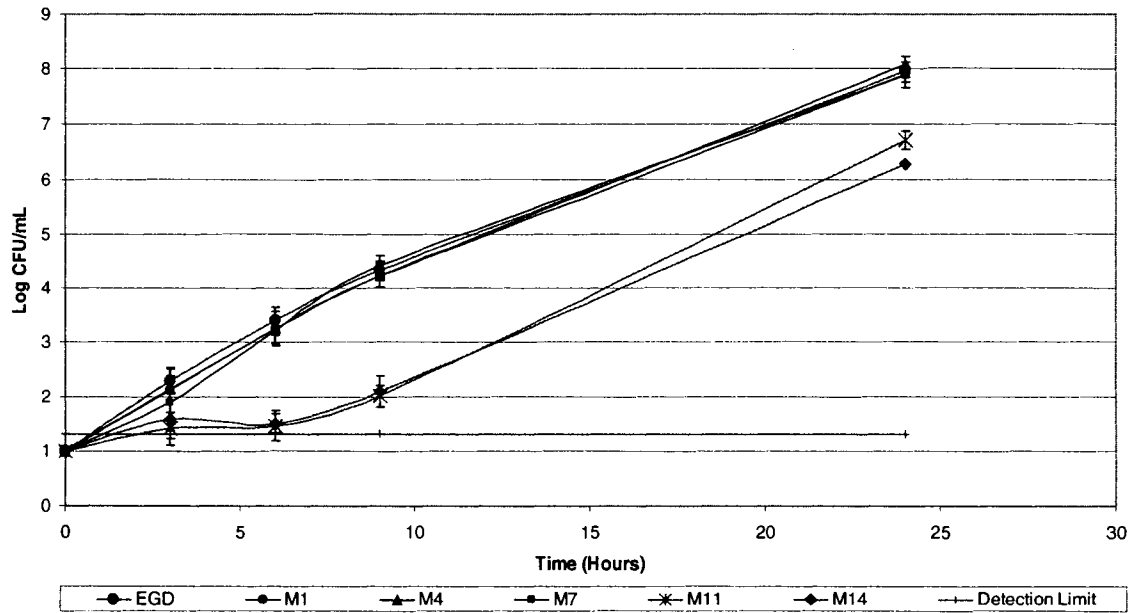


Fig. 2.1-b. Growth curves for *Listeria monocytogenes* wild-type EGD and its mutants at 22°C



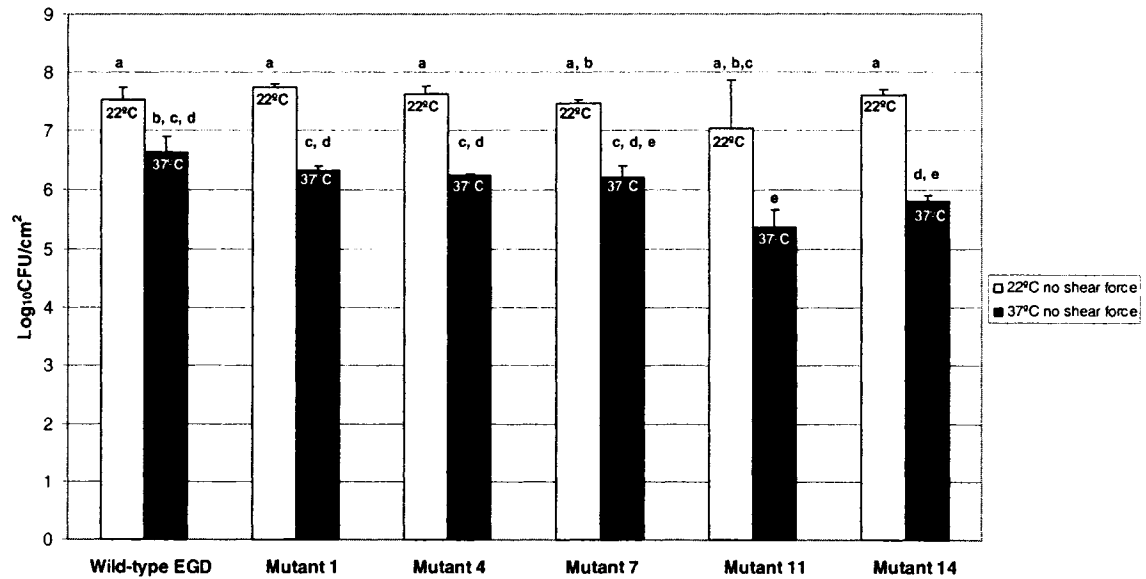


Fig. 2.2-a. Average bacterial cell counts & st. dev. of surface associated *L. monocytogenes* EGD & its corresponding mutant strains at 22°C and 37°C without shear force

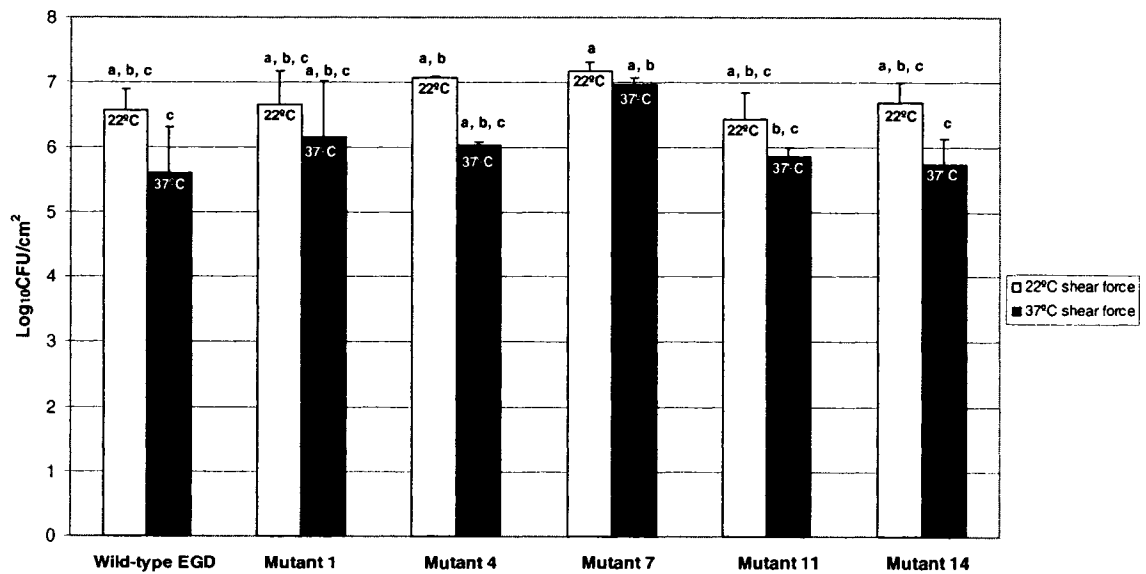


Fig. 2.2-b. Average bacterial cell counts & st. dev. of surface associated *L. monocytogenes* EGD & its corresponding mutant strains at 22°C and 37°C with shear force

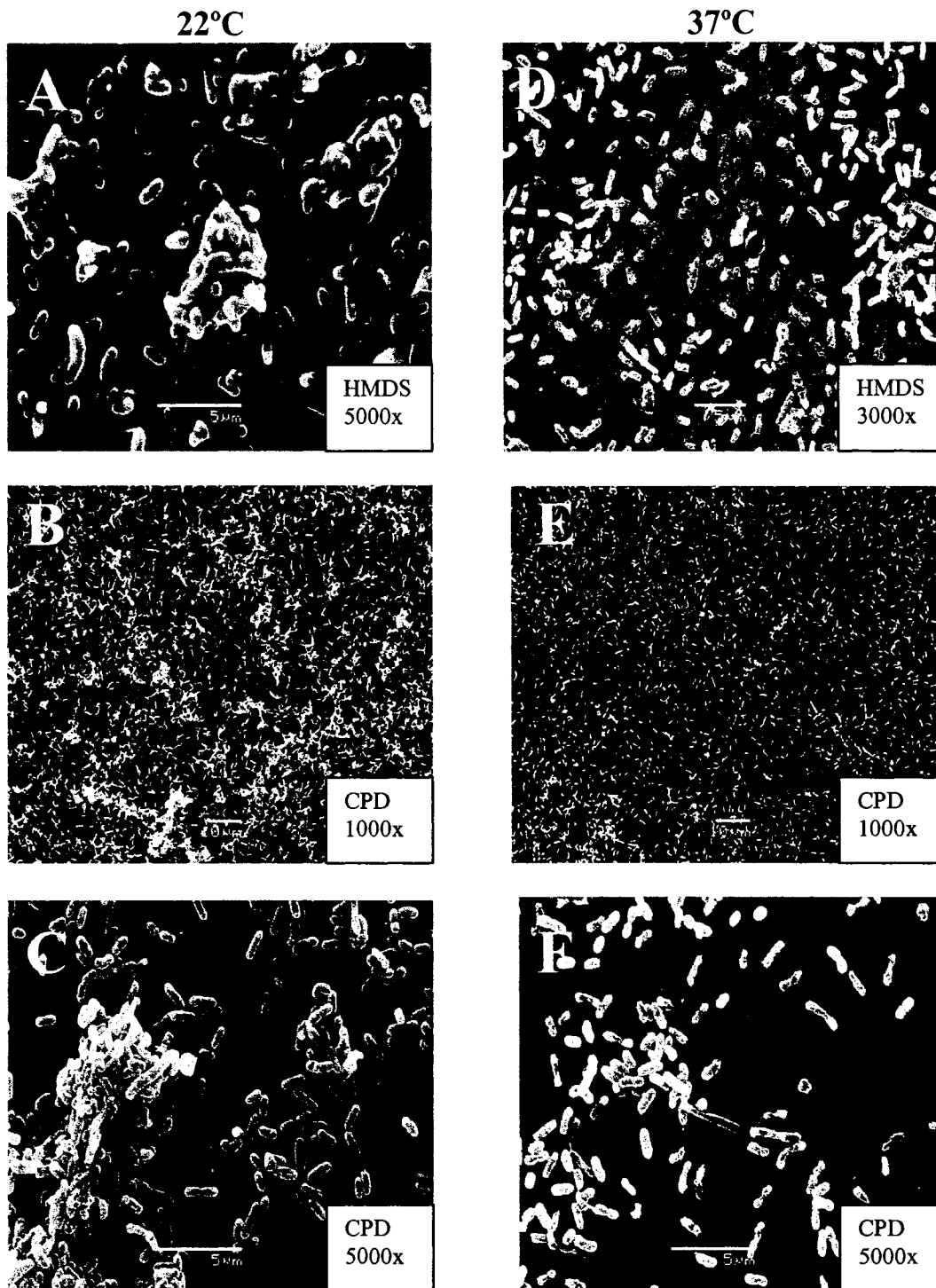


Fig. 2.3-a. SEM images of *Listeria monocytogenes* mutant strain M11 at (A) 5000x, (B) 1000x, (C) 5000x, (D) 3000x, (E) 1000x, (F) 5000x magnification. Biofilms were grown at 22°C (A, B, C) and 37°C (D, E, F) for 24 hours. Biofilms were subjected to HMDS (A, D) and CPD (B, C, E, F) before viewing with SEM. Bar = 5 $\mu$ m (A, C, D, F). Bar = 10 $\mu$ m (B, E)

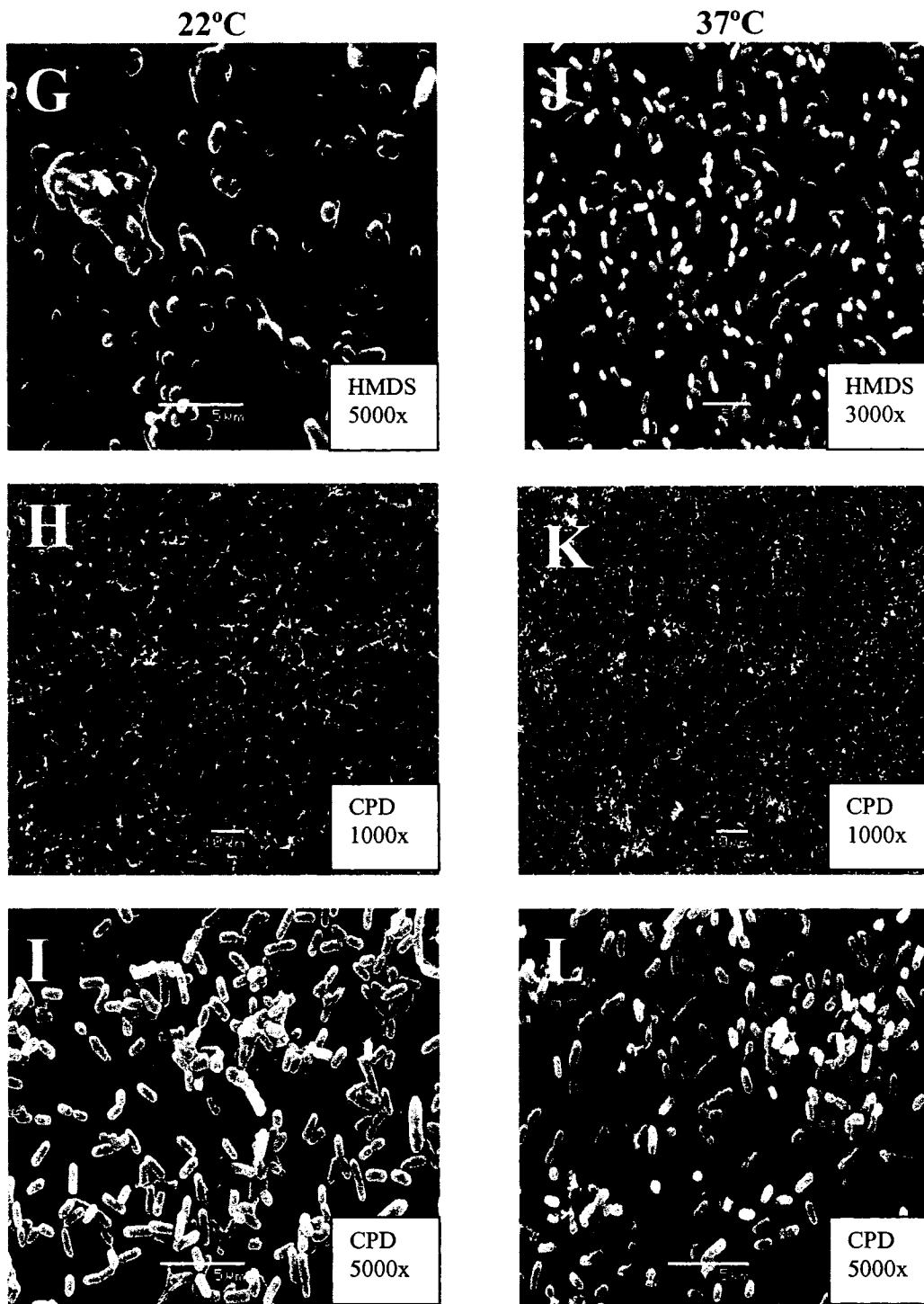


Fig. 2.3-b. SEM images of *Listeria monocytogenes* strain EGD at (G) 5000x, (H) 1000x, (I) 5000x, (J) 3000x, (K) 1000x, (L) 5000x magnification. Biofilms were grown at 22°C (G, H, I) and 37°C (J, K, L) for 24 hours. Biofilms were subjected to HMDS (G, J) and CPD (H, I, K, L) before viewing with SEM. Bar = 5µm (G, I, J, L). Bar = 10µm (H, K)

## References

- Botes, L, B. Price, M. Waldron, and Pitcher, G. C. 2002. A simple and rapid scanning electron microscope preparative technique for delicate “Gymnodinioid” dinoflagellates. *Microsc. Res. Tech.* 59:128–130.
- Boyde, A. and Wood, C. 1969. Preparation of animal tissues for surface-scanning electron microscopy. *J. Microsc.* 90:221-249.
- Braet, F., R. De Zanger, and Wisse, E. 1997. Drying cells for SEM, AFM and TEM by hexamethyldisilazane: A study on hepatic endothelial cells. Pt 1. *J. Microsc.* 186:84-87.
- Bray, D. F., J. Bagu, and Koegler, P. 1993. Comparison of Hexamethyldisilazane (HMDS), Peldri II, and critical point drying methods for scanning electron microscopy of biological specimens. *Microsc. Res. Technique*, 26:489-495.
- Carpentier, B. and Cerf, O. 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Microbiology* 75:499-511.
- Chae, M. S. 2004. The role of flagella, physicochemical surface properties, and genes of *Listeria monocytogenes* in attachment and biofilm formation. PhD Thesis. Department of Food Science. University of Guelph.
- Cooley, W. A., A. C. Scott, and Simmons, M. M. 1994. The use of falcon cell culture inserts and Hexamethyldisilazane for the study of small biological specimens by electron microscopy. *Eur. Microsc. Analysis*, 31:17-19.
- Cortizo, M. C. and de Mele, M. F. L. 2003. Microstructural characteristics of thin biofilms through optical and scanning electron microscopy. *J. Microbiol. and Biotechnol.* 19:805-810.
- Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and Lappin-Scott, H. M. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* 49:711-745.
- Daud, H. B., T. A. McMeekin, and Olley, J. 1978. Temperature function integration and the development and metabolism of poultry spoilage bacteria. *App. Environ. Microbiol.* 36:650-654.
- Dekker, N. P., C. J. Lammel, and Brooks, G. F. 1991. Scanning electron microscopy of piliated *Neisseria gonorrhoeae* processed with Hexamethyldisilazane. *J. Electron Microsc. Technique* 19:461-467.
- Dey, S., T. S. Basu-Baul, B. Roy, and Dey, D. 1989. A new rapid method of air drying for scanning electron microscopy using tetramethylsilane. *J. Microsc.* 156:259–261.

- Donlan, R. M. 1992. Biofilms: microbial life on surfaces. *Emerg. Inf. Dis.* 8(9):881-890.
- Donlan, R. M. and Costerton, J. W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15(2):167-193.
- Dons, L., O. F. Rasmussen, and Olsen, J. E. 1992. Cloning and characterization of a gene encoding flagellin of *Listeria monocytogenes*. *Mol. Microbiol.* 6:2919-2929.
- Forge, A., G. Nevill, G. Zajic, and Wright, A. 1992. Scanning electron microscopy of the mammalian organ of Corti: assessment of preparative procedures. *Scanning Microsc.* 6:521-535.
- Gjaltema, A., P. A. M. Arts, M. C. M. van Loosdrecht, J. G. Kuenen, and Heijnen, J. J. 1994. Heterogeneity of biofilms in rotating annular reactors: Occurrence, structure and consequences. *Biotechnol. Bioeng.* 44:194-204.
- Gjaltema, A., L. Tjihuis, M. C. M. van Loosdrecht, and Heijnen, J. J. 1995. Detachment of biomass from suspended nongrowing spherical biofilms in airlift reactors. *Biotechnol. Bioeng.* 46:258-269.
- Liu, Y. and Tay, J. H. The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. 2002. *Water Res.* 36(7):1653-65.
- Liu, Z., V. Tek, V. Akoev, and Zolkiewski, M. 2002. Conserved amino-acid residues within the amino-terminal domain of *clpB* are Essential for the chaperone activity. *J. Mol. Biol.* 321:111-120.
- Loosdrecht, van M. C. M., J. J. Heijnen, H. Eberl, J. Kreft, and Picioreanu, C. 2002. Mathematical modelling of biofilm structures. *Antonie van Leeuwenhoek* 81:245-256.
- McCoy, W. F., J. D. Bryers, and Costerton, J. W. 1981. Observations of fouling biofilm formation. *Can. J. Microbiol.* 27:910-917.
- Nation, J. L. 1983. A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Stain Technol.* 58:347-351.
- Peel, M., W. Donachie, and Shaw, A. 1988. Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE, and western blotting. *J. Gen. Microbiol.* 134:2171-2178.
- Ploeg, van der J. R., E. Eichhorn, and Leisinger, T. 2001. Sulfonate-sulfur metabolism and its regulation in *Escherichia coli*. *Arch. Microbiol.* 176(1-2):1-8.
- Ronner, A. B. and Wong, A. C. L. 1993. Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-N rubber. *J. Food Prot.* 56:750-758.

- Rupp, C. J., C. Fux, and Stoodley, P. 2005. Viscoelasticity of *Staphylococcus aureus* biofilms in response to fluid shear resists detachment and facilitates rolling migration. *Appl. Environ. Microbiol.* 71(4):2175-2178.
- Sasahara, K. and Zottola, E. A. 1993. Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. *J. Food Prot.* 56:1022-1028.
- Stewart, P. S., G. A. Mcfeters, and Huang, C. 2000. Biofilm control by antimicrobial agents. In *Biofilms II: Process Analysis and Applications*, Bryers, D. ed. pp. 375-405.
- Stoodley, P., Z. Lewandowski, J. D. Boyle, and Lappin-Scott, H. M. 1999. Structural deformation of bacterial biofilms caused by short term fluctuations in flow velocity: an in situ demonstration of biofilm viscoelasticity. *Biotechnol. Bioeng.* 65:83-92.
- Tijhuis, L., B. Hijman, M. C. M. van Loosdrecht, and Heijnen, J. J. 1996. Influence of detachment, substrate loading and reactor scale on the formation of biofilms in airlift reactors. *Appl. Microbiol. Biotechnol.* 45:7-17.
- Van Alstyne, D., G. F. Grant, and Simon, M. 1969. Synthesis of bacterial flagella: Chromosomal synchrony and flagella synthesis. *J Bacteriol.* 100(1):283-287.
- Vatanyoopaisarn, S., A. Nazli, E. R. Dodd, E. D. Rees, and Waites, W. M. 2000. Effect of flagella on initial attachment of *L. monocytogenes* to stainless steel. *Appl. Env. Microbiol.* 66(2):860-863.
- Verschuren, P. G. and van den Heuvel, J. C. 2002. Substrate controlled development of anaerobic acidifying aggregates at different shear rates in a gas lift reactor. *Biotech. Bioeng.* 77:306-315.
- Way, S. S., L. J. Thompson, J. E. Lopes, A. M. Hajjar, T. R. Kollman, N. E. Freitag, and Wilson, C. B. 2004. Characterization of flagellin expression and its role in *Listeria monocytogenes* infection and immunity. *Cell Micro.* 6(3):235-242.
- Wirtanen, G. and Mattila-Sandholm, T. 1992. Effect of the growth phase of foodborne biofilms on their resistance to a chlorine sanitizer. Part II. *Lebesm. Wiss. U. Technol.* 25:50-54.
- Zottola, E. A. and Hood, S. K. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. 1997. *Int. J. Food Microbiol.* 37(2-3):145-153.

## Chapter Three

### Characterizing Important Biofilm Structures Produced by Wild type and Mutant Strains of *Listeria monocytogenes* by PCA (Principal Components Analysis)

#### 3.1. INTRODUCTION

When bacteria attach and colonize a surface, they form a community called a biofilm. The formation of biofilms creates major problems for food industries as bacteria in a biofilm are less susceptible to disinfectants (Ronner and Wong, 1993; Sagripanti and Bonifacino, 2000), have increased exopolysaccharide production (Sutherland 2001), and can contaminate food processing equipment (Carpentier and Cerf, 1993; Carpentier *et al.*, 1998). When describing a biofilm, structural characteristics come to mind. For this, many studies have employed confocal scanning laser microscopy (CSLM) to obtain depth-selective information on the three-dimensional structure of biofilms (Costerton *et al.*, 1995; DeBeer *et al.*, 1994).

Descriptive CSLM studies of microbial biofilms can only provide limited information on biofilm architecture. For this reason, researchers incorporate the use of novel computer programs such as COMSTAT or Phobia LSM Image Processing tool (PHLIP) to describe/quantify biofilm structures (Heydorn *et al.*, 2000; Mueller, 2004). The use of novel computer programs can generate large amounts of data regarding selected biofilm structures which can be difficult to interpret or compare when many bacterial strains are studied. Thus, statistical methods can be employed to characterize these structures for comparison. We have chosen to employ in this study principal components analysis (PCA), which has been widely used in all areas of Ecology and Systematics. To date, very few reports on its use in the area of Microbiology are available that probe into foodborne microbial biofilms. PCA reduces the dimensions of a single group of data by producing a smaller number of abstract variables (linear combinations of the original variables, principal components). The method is based on maximization of the variance of linear combinations of variables. Successive components are constructed

to be uncorrelated with previous ones. Often, most of the variation can be summarized with only a few components, so data with many variables can be displayed effectively on a two or three-dimensional graph that uses the components as axes (Digby and Kempton, 1987; Harris, 1985; Johnson and Wichern, 1988; Neff and Marcus, 1980; Pielou, 1984). Thus, biofilm structures from various bacterial strains (quantified by novel computer programs) can be separated and grouped together based on similar values graphically. In addition, important variables extracted from the PCA allow one to subsequently employ other statistical techniques to investigate any differences between strains.

The objectives of this study were to (1) analyze wild-type *Listeria monocytogenes* EGD and five of its corresponding mutant strains for their ability to form biofilms on glass under various conditions (22°C and 37°C with and without shear force), (2) use confocal scanning laser microscopy (CSLM) to analyze biofilm architecture of wild-type *L. monocytogenes* EGD and its corresponding mutant strains, (3) quantitatively describe with PHLIP - biofilm total biovolume, surface to biovolume ratio, mean thickness and roughness, and (4) use PCA and subsequent statistical methods to compare and analyze important biofilm structures produced by *L. monocytogenes* wild-type and mutant strains.

## **3.2. MATERIALS AND METHODS**

### **3.2.1. Bacterial Strains**

Bacterial strains used in this study were the same as described in Chapter 2.

### **3.2.2. Cultures and growth conditions**

Cultures and growth conditions were the same as described in Chapter 2.



### **3.2.3. Growth of Biofilms on Glass Cover Slips**

#### **3.2.3.1. Preparation of glass Petri dishes and cover slips**

Glass cover slips (Fisher premium 24 x 60mm) and glass Petri dishes (100mm diameter) were washed by a 10 minute immersion in 1000mL of an aqueous 2% RBS Detergent Concentrate solution at 50°C (20mL of RBS 35 Concentrate per liter of tap water; Pierce, Rockford, IL), and rinsed by immersion in 1L of tap water (initial temp. 50°C) with agitation for 25 minutes. Following that, five additional 1 minute immersions with agitation in 1L of distilled water at ambient temperature were performed. Lastly, each glass cover slip was placed in a Petri dish, and the assembly autoclaved at 121°C for 20 minutes.

#### **3.2.3.2. Formation of biofilms**

Standardized cell suspensions were prepared for each strain as described in Chapter 2, and 12mL deposited into each sterilized glass Petri dish covering the glass cover slip. The dishes were then closed and placed in larger glass Petri dishes (Pyrex glass petri dish 15cm diameter) containing moist paper towels which provided humidity during incubation at 37°C for 3 hours to allow for adhesion to occur. After the 3 hour adhesion period, the non-adherent bacteria were removed by slowly pipetting 50mL (25mL x 2) of PBS over the bottom of each of the smaller Petri dishes containing the cover slips. Next, 12mL of TSB was placed over the adherent cells and the dishes were again placed in the larger glass Petri dishes for a 24 hour incubation period at 4 different treatment levels: 22°C and 37°C, with and without shear force. After 1 day of incubation, the glass cover slips were rinsed as described above to remove any non-adherent bacteria. The biofilms were fixed in 4% paraformaldehyde for one hour and then stained with 10µg/ml propidium iodide (Molecular Probes, Eugene, OR, USA) for 30 minutes. Excess propidium iodide was gently rinsed off with distilled water. A Press-to-Seal silicon isolater (20mm dia. x 1mm depth, Molecular Probes, Eugene, OR, USA) was next placed

over the middle surface of the biofilm-covered glass cover slips after placing a drop of distilled water in the middle of each cover slip. A glass slide was next sealed to the other side of the Press-to-Seal silicon isolater and the biofilm was viewed inverted on the confocal scanning laser microscope (CSLM). For each strain and treatment level, three replicates were performed.

#### **3.2.4. Image Acquisition**

All microscopic observations and image acquisitions were performed by an Olympus CSLM (FV300; Olympus America Inc., Melville, NY, USA). Images were obtained with a 60x (1.4 NA) oil immersion lens and detected using green laser (530-550 nm excitation) from an Ar-Kr mixed gas laser. For each 24h biofilm sample, five randomly selected fields of view were analyzed by taking sections in 0.1 to 0.5  $\mu\text{m}$  intervals down through the biofilm. Thus, the number of images in each stack varied according to the thickness of the biofilm. Files from each image stack were saved and later used for image analysis by the Phobia LSM Image Processing tool (PHLIP).

#### **3.2.5 Image Analysis by PHLIP**

Following acquisition by CSLM, images were quantified by the PHLIP program, which calculates a wide range of variables describing biofilm structures, such as total biovolume, substratum coverage, surface area to biovolume ratio, mean thickness, roughness, spatial spreading of biovolume and fractal dimension in 2D. Mainly implemented as a script in MATLAB 5.1 (The MathWorks), each image stack is first thresholded using automated threshold determination which is supported by a graphical user interface (GUI). In the GUI, the determined threshold levels for a given stack are displayed. Stacks where low threshold values (lower than 20) were found, were adjusted manually as described in the PHLIP homepage at

<http://www.itqb.unl.pt:1141/~webpages/phlip/>. For this study, total biovolume, surface area to biovolume, mean thickness and roughness were selected as analysis parameters.

### 3.2.6. Statistical Analysis

Biofilm structures quantified by PHLIP were analyzed using principal components analysis (PCA). A one way analysis of variance (ANOVA) and Tukey's HSD Post Hoc Multiple Comparison test were then employed to further analyze all important variables influencing biofilm structure extracted from the PCA for between strain differences. Following that, a two way analysis of variance was performed to look into the treatment effects of temperature, shaking and strain on these important biofilm structures. All calculations were performed using Statistical Package for the Social Sciences (SPSS) for Windows version 11.0.1 (SPSS Inc., Chicago, Illinois) and statistical difference was evaluated at  $P < 0.05$ .

## 3.3. RESULTS

### 3.3.1. PHLIP analysis of *L. monocytogenes* Biofilm Structures

Results from the PHLIP analysis of biofilm total biovolume (Fig. 3.1-a) found that mutant strain M7 had the highest mean biovolume compared to all other strains when grown at 22°C without shear force ( $52484.10 \pm 3448.11 \mu\text{m}^3$ ) and 37°C with shear force ( $37361.96 \pm 5637.81 \mu\text{m}^3$ ). *L. monocytogenes* wild-type strain EGD on the other hand, was observed having the highest mean biovolume when grown at 22°C with shear force ( $33026.48 \pm 8051.56 \mu\text{m}^3$ ) and at 37°C without shear force applied ( $30336.23 \pm 7865.09 \mu\text{m}^3$ ). Mutant strains M11 and M14 had the lowest mean biovolume compared to all other strains investigated for 3 of the treatments (22°C with shear force, 37°C with and without shear force). M14 had the lowest mean biovolume amongst all other strains when grown at 22°C without shear force ( $12320.73 \pm 6917.85 \mu\text{m}^3$ ).

Analysis of surface area to biovolume ratio (Fig. 3.1-b) found mutant strain M7 having the lowest means compared to all other strains, across three treatment levels (22°C without shear force, 37°C with and without shear force applied). On the other hand, EGD, mutant M1 and M11 biofilms had the highest means when grown at 22°C without shear force, 37°C with shear force and 37°C without shear force respectively. Biofilms grown at 22° with shear force applied resulted in mutants M4 and M14 having the lowest ( $4.46 \pm 0.23 \mu\text{m}^{-1}$ ) and highest ( $8.61 \pm 1.34 \mu\text{m}^{-1}$ ) mean surface area to biovolume ratios respectively.

The PHLIP analysis of biofilm thickness (Fig 3.1-c) showed that *L. monocytogenes* EGD and mutant M1 had low thickness means compared to all other strains with respect to all treatment levels. M1 had the lowest biofilm thickness means for 3 out of 4 treatment levels – 22°C with shear force, 37°C with and without shear force. Wild-type EGD had the lowest mean biofilm thickness when grown at 22°C without shear force ( $1.47 \pm 0.044 \mu\text{m}$ ). Mutant M7 was observed having the highest mean biofilm thickness when grown at 22°C with and without shear force applied ( $3.3 \pm 0.76 \mu\text{m}$  and  $3.24 \pm 0.06 \mu\text{m}$ ). On the other hand, mutant M11 was seen having the highest mean biofilm thickness when grown at 37°C with and without shear force ( $2.82 \pm 0.28 \mu\text{m}^3$  and  $3.44 \pm 0.59 \mu\text{m}$ ) applied.

The PHLIP analysis of biofilm roughness (Fig 3.1-d) resulted in *L. monocytogenes* wild-type EGD having the highest mean roughness compared with all other strains at 22°C with shear force ( $0.50 \pm 0.061$ ) and 37°C without shear force ( $0.50 \pm 0.0078$ ) applied. Mutant M7 was also found having the highest mean roughness compared to all other strains when grown at 22°C without shear force ( $0.51 \pm 0.0031$ ) and 37°C with shear force ( $0.49 \pm 0.023$ ). In contrast, mutants M14 and M11 had the lowest mean roughness at 22°C without shear force ( $0.35 \pm 0.077$ ) and at 37°C with shear force ( $0.067 \pm 0.018$ ) respectively. In addition, Mutants M7 and M14 were observed having low roughness means at 22°C with shear force ( $0.051 \pm 0.0064$ ) and 37°C without shear force ( $0.071 \pm 0.020$ ) respectively.

### **3.3.2. One-way ANOVA and Tukey's HSD Post Hoc Multiple Comparison Test on *L. monocytogenes* Biofilm Structures**

A one-way analysis of variance was used to compare strains groups and important variables extracted from PCA (Table 3.1-a). Biovolume, surface area to biovolume ratio, thickness and roughness means differed across all strain groups ( $P < 0.05$ ).

A Tukey's HSD Post Hoc Multiple Comparison test was performed to determine which strain group differed with respect to each biofilm structure investigated (Table 3.1-b). Mutant M14 differed significantly from wild-type strain EGD, mutants M4 and M7, by having a low mean biovolume. In contrast, wild-type strain EGD and mutant M7 differed significantly from mutants M1, M11 and M14 because of high biovolume means.

The test on surface area to biovolume ratio showed mutant M4 differing significantly from strains M1, M11 and M14. Mutant M7 differed from M1, whereas mutant M1 differed significantly from M4 and M7. Mutants M4 and M7 were low in mean surface area to biovolume whereas M1 was high.

As for biofilm mean thickness, mutant M1 was found to be significantly different from mutants M4, M7, M11 and M14, because of low mean thickness. Mutant M11 differed significantly from strains EGD, M1 and M4 by having higher mean thickness. Mutants M7 and M14 also differed from strains EGD and M1, because of higher mean thickness.

Biofilm mean roughness on the other hand, showed mutants M11 and M14 differing significantly from all other strains in study. Mutants M11 and M14 differed from all other strains because of low mean roughness.

### **3.3.3. Principal components analysis of *L. monocytogenes* biofilm structures**

Principal component analysis (PCA) was used to explain variability in biofilms of *L. monocytogenes* wild-type strain EGD and its mutants in fewer dimensions. PCA of the 2-D data - a matrix of 72 rows (observations, i.e., PHLIP values) and 12 columns (variables, i.e., incubation conditions and biofilm structures), in which observations were

standardized horizontally, extracted four principal components (PCs) accounting for 87.70% of the total variability in biofilm structure. As shown in Table 3.2-a, principal components 1 and 2 captured most of the variability among biofilms. The first principal component (PC1) explained 58.16% of variation in data, whereas the second principal component (PC2) explained 29.54%. Based on factor loadings (Table 3.2-b), the variables most highly associated with PC1 were biovolume, surface area to biovolume ratio, and roughness. PC2 on the other hand, was highly associated with surface area to biovolume ratio and thickness. A scatter plot of PC1 (first axis) x PC2 (second axis) with strains and their individual growth conditions set as markers, showed some clustering of the 6 strains used in the study which revealed several interesting trends (Fig. 3.2).

For the wild-type strain *L. monocytogenes* EGD and its mutant M4, all four treatments placed in the centre of the scatter plot and had thus no unique characteristics with respect to biofilm thickness, roughness, biovolume and surface area to biovolume ratio (S/Bv).

Similar observations applied to three treatments (22°C-S, 22°C-NS, and 37°C-NS) of mutant M1, but biofilms grown at 37°C with shear force were separated from other strain-treatment combinations due to low thickness.

Another unique cluster was characterized by biofilms high in thickness and S/Bv, but low in biovolume and roughness. This cluster comprised three strains (M14, M11 and M7). For M14 and M11, biofilms grown in three conditions (22°C-S, 37°C-S, and 37°C-NS) placed in this cluster. Biofilms of these two strains grown at 22°C-NS placed either in the centre of the scatter plot (M14) or featured relatively high thickness (M11). For M7, only biofilms grown at 22°C with shear force placed into the unique cluster (high in thickness and S/Bv, but low in biovolume and roughness). Strains of mutant M7 grown at 22°C without shear force, clustered with high thickness, high biovolume and high roughness, while biofilms of strain M7 grown at 37°C (with and without shear force) placed in the centre of the scatter plot.

### 3.3.4. Two-way ANOVA on Factors Affecting *L. monocytogenes* Biofilm Structure

A two-way analysis of variance was performed to examine the effects of 2 different treatments on biofilm structures. From results, the interaction between strain and temperature had a significant effect on mean surface area to biovolume and roughness (Table 3.3-a). The interaction between shear force and temperature significantly affected mean biovolume and roughness (Table 3.3-b). In addition, mean biovolume, surface area to biovolume, and roughness was significantly affected by the interaction between strain type and shear force (Table 3.3-c). It was also observed that temperature, on its own, had no effect on any biofilm structures in the study (Table 3.3-a, 3.3-b). However, strain type did have a significant effect on all biofilm structures investigated (Table 3.3-a, 3.3-c). Shear force was also seen having a significant effect on both mean biovolume and roughness (Table 3.3-b, 3.3-c). When explored further with box plots, it was found that biovolume and roughness means were higher for biofilms produced without shear force than with shear force applied (Fig. 3.3-a, 3.3-b).

## 3.4. DISCUSSION

*L. monocytogenes* EGD and its five mutants readily developed biofilms on glass cover slips when incubated at 22°C and 37°C with and without shear force applied. In this study, shear force and strain type were observed having a significant effect on many biofilm structures of *L. monocytogenes* wild-type EGD and its mutants. Mutant M7 formed consistently thicker and rougher biofilms that were also high in biovolume compared to all other strains. From results of the PCA, mutant M7 grown at 22°C without shear force, produced biofilms with high biovolume, thickness and roughness. These findings also conferred with results from the one-way analysis of variance. Also, because temperature had no significant effect on biofilm structure means for all strains, the interaction between shear force and temperature in significantly affecting biovolume and roughness, was more likely due to shear instead. As seen from box plots from two-way ANOVA results, growing mutant M7 without shear force probably aided to increase its

roughness and biovolume potential. However, the effect of shear force or temperature had no significant effect on thickness for mutant M7. Thus, increased thickness could only be attributed to strain type as seen from two-way ANOVA results. Mutant M7 has a transposon insertion within the *lmo2435* gene, which was found to be similar to the *B. subtilis* YfhL protein. The function of this protein is not yet known and few studies have actually looked into the biofilm forming capabilities of *yfhL* mutants. Although our studies show that the inactive *yfhL* gene in mutant M7 might have played a role in the biofilm structures formed, further studies are still required to elucidate the actual role of this gene in biofilm formation.

Mutant M14 was unique in the sense that it formed consistently thicker and higher surface area to biovolume biofilms compared to all other strains. This phenomenon was observed for this strain produced at 37°C and 22°C, with shear force and at 37°C without shear force applied. Like mutant M7, increased thickness was most probably attributed to strain type as seen from two-way ANOVA results. This finding also confers with results from the one-way ANOVA. In addition, a high surface area to biovolume ratio seen for M14 was probably due to interactions between shear force and strain or strain and temperature. Because shear force or temperature did not have any significant effect on surface area and biovolume ratio, it can thus be said that strain type played a more significant role in the interactions between temperature or shear force. The same can be said for mutant M11 produced at 37°C with shear force applied. Mutant M14 had a transposon insertion within the terminator of the *cysK* gene, suggesting the possibility that the *L. monocytogenes* *cysK* gene was not well expressed in this mutant. *CysK* in *B. subtilis* is thought to encode *O*-acetylserine lyase A, which is involved in cysteine biosynthesis since it shows 43% amino acid sequence identity to the *cysK* gene of *E. coli* (van der Pleog *et al.*, 2001). Interestingly, *cysK* was previously identified in a search for *E. coli* genes regulated by quorum sensing (Baca-DeLancey *et al.*, 1999) and the activation of *cysK* may reflect a requirement for increased cysteine in stationary-phase cells. Baca-DeLancey *et al.* (1999) developed a screen for the identification of quorum sensing regulated genes in *E. coli* and found that the quorum sensing activated *cysK*, *astCADBE*, *tnaAB*, and *gabDTP* function in the uptake, synthesis, and catabolism of amino acids to generate pyruvate or succinate. This may be important for energy



production in stationary phase. Thus, a high surface area to biovolume ratio seen for mutant M14 could be due to its inability to catalyze cysteine in nutrients and had to increase its biofilm surface area to biovolume ratio to optimize access to more nutrients due to this shortage.

Mutant M11 had a transposon insertion in the *clpB* gene. *ClpB* belongs to the *Clp* ATPase protein family, which comprises *clpA*-, *clpB*-, *clpC*-, and *clpX*-like proteins and includes several stress response proteins (Squires and Squires, 1992). Conditions which induce expression of the *clp* stress proteins include high temperature, high salt or ethanol concentration, oxygen limitation, and iron limitation. Bacterial *clp* mutants have shown increased sensitivity to a range of stress conditions in vitro (Kruger *et al.*, 1994; Squires *et al.*, 1991; Squires and Squires, 1992; Rouquette *et al.*, 1996); e.g., *Listeria monocytogenes clpC* mutants which are sensitive to high temperature, high osmolarity, iron limitation, and oxidative stress and are attenuated in mice (Rouquette *et al.*, 1996). In *E. coli*, *clpB* is unique among the Hsp100 proteins (involved in numerous cellular activities that rely on ATP binding and hydrolysis by AAA+ proteins), as it mediates the solubilization and refolding of aggregated proteins in cooperation with the DnaK chaperone system (DnaK, DanJ, GrpE: KJE) (Neuwald *et al.*, 1999; Goloubinoff *et al.*, 1999; Mogk *et al.*, 1999; Zolkiewski, 1999). *ClpB* is involved in maintaining cell viability after heat shock in *E. coli* and is essential for the development of thermal tolerance in *H. pylori*, *Synechococcus* spp., *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* (Hong and Vierling, 2000; Allan *et al.*, 1998; Eriksson and Clarke, 1996; Sanchez and Lindquist, 1990). Allan *et al.* (1998) investigated the the role of the *clpB* gene product in *H. pylori*. They found that the viable count for the *clpB* mutant was lower than that of the wild-type strain at each subsequent sampling time at 50°C and no difference in the rate of growth was apparent between mutant and wild-type bacteria on *Helicobacter* selective agar at 30, 37, or 40°C. *ClpB* is also involved in cold acclimation (Porankiewicz and Clarke, 1997). *ClpB* induction plays a critical role in cold acclimation in the cyanobacterium *Synechococcus* sp. strain PCC7942 (Porankiewicz and Clarke, 1997). A study by Liu *et al.* (2002) found that increased expression of mRNAs for *clpB* was induced in *L. monocytogenes* growing at 10°C relative to those growing at 37°C. Increased expression of mRNAs for the chaperone protease *clpB* may reflect the

production of improperly folded or otherwise damaged proteins arising during *L. monocytogenes* growth at low temperatures due to cold shock. The stress responses in bacteria vary in accordance with the nature of the stress. Stress response genes for e.g. *rpoS* have been reported to control quorum-sensing genes, which are considered responsible for cell-cell communication in bacteria. *RpoS* is seen as a master regulator of stress responses and in many circumstances, may provide bacterial cells with the ability to tolerate actual as well as additional stresses (Hengge-Aronis, 2002). Corona-Izquierdo and Membrillo-Hernandez (2002) reported that a mutation in *rpoS* in *E. coli* enhanced the formation of biofilm. In another study by Nandakumar *et al.* (2006), it was found that a *Moraxella* mutant (*rpoS* knocked out) formed significantly denser and thicker biofilms than its wild-type parent. Biofilm formation is considered to be a process of slow growth and a mechanism for dealing with stressful conditions. Thus, stress response genes such as *clps* and *rpoS* could be thought of as playing major roles in biofilm formation. In our study, mutant M11 (*clpB* inactive) was observed forming biofilms with high thickness and surface to biovolume ratio, which agrees with observations found by other researchers in regard to stress response genes. In addition, since temperature did not play a significant role in biofilm formation for the *clpB* mutant, we could probably assume that the significance of the structures produced by mutant M11 was caused by the absence of the stress response *clpB* gene.

Mutant M1 was unique in that it portrayed very low thickness compared to all other strains when grown at 37°C. It was found as a single cluster with two other strains (outliers) in principal components space. Mutant M1 had a transposon insertion of Tn916 located 26 bp upstream from a 1200 bp ORF similar to the *dfp* (flavoprotein) gene in *B. subtilis* (60% identity at the amino acid level). Milošević *et al.* (2000) performed insertional mutagenesis to obtain a mutant with Tn1545 inserted just upstream from the *dfp* and *priA* of *L. monocytogenes*. They determined that a ribosome binding site (RBS) was located 15 nucleotides upstream from the start codon for the *L. monocytogenes dfp*, but no potential RBS was detected between the two genes. They suggested that the *dfp* and *priA* genes may be translated together and be involved in the adhesion of *L. monocytogenes* to eukaryotic cells. In another study, Polard *et al.* (2002) found that the disruption of the *priA* gene in *B. subtilis* (involved in primosome formation), resulted in

decreased cell viability and growth, caused filamentation and increased sensitivity to rich media and UV light. This could be the case for decreased thickness seen for mutant M1 since mean biofilm thickness is a measure of the spatial size of a biofilm.

As for wild-type strain EGD and mutant M4, no distinct characteristics in biofilm structure were observed. Even though mutant M4 had a transposon insertion in the promoter region of the chemotaxis *cheYA* operon, they were clustered together in principal components space with their wild-type parent, forming biofilms of similar structure. Transposon mutations in the promoter region of the *cheYA* operon of *L. monocytogenes* have been shown to reduce flagellin expression and mutants were unable to swim on soft agar plates, and appeared deficient in their ability to attach to the mouse fibroblast cell line 3T3 (Flanary *et al.*, 1999). In addition, studies by Pratt and Kolter (1998) reported that chemotaxis in *E. coli* was important for the initial stages of biofilm development on abiotic surfaces. This however, does not seem to be the case for mutant M4 in this study as this strain performed equally as well as its parent. Thus, further studies have to be done to reassess the adhesion and biofilm forming capabilities of mutant M4 on various other substrates.

Our present investigation into biofilm structures with CSLM and the subsequent analysis with PHLIP, along with various statistical methods have proved useful in characterizing strain types with regard to biofilm structures produced under different conditions. Although many of the processes regarding biofilm formation by *Listeria* transposon mutants seem unclear, we are certain that further investigations will bring about greater insights into the genetic processes regarding biofilm production and structure formation in *L. monocytogenes*.

**Table 3.1-a. One-way ANOVA results**

		<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>Sig.</b>
<b>Biovolume</b>	Between Groups	6127545873.811	5	1225509174.762	9.291	.000
	Within Groups	8705374857.565	66	131899619.054		
	Total	14832920731.376	71			
<b>S/Bv Ratio</b>	Between Groups	63.773	5	12.755	4.791	.001
	Within Groups	175.712	66	2.662		
	Total	239.484	71			
<b>Thickness</b>	Between Groups	16.905	5	3.381	13.497	.000
	Within Groups	16.533	66	.250		
	Total	33.438	71			
<b>Roughness</b>	Between Groups	1.104	5	.221	13.592	.000
	Within Groups	1.072	66	.016		
	Total	2.176	71			

**Table 3.1-b. Tukey's HSD Post Hoc Multiple Comparison test results**

**Biovolume**

	N	Subset for alpha = .05		
STRAIN		1	2	3
M14	12	3593.55406		
M11	12	8965.52651	8965.52651	
M1	12	13345.39147	13345.39147	
M4	12	20439.45295 20439.45295		
EGD	12	27359.43727		
M7	12	28569.43074		
Sig.		.310	.155	.515

**Surface area to biovol. ratio**

	N	Subset for alpha = .05		
STRAIN		1	2	3
M4	12	4.90610		
M7	12	5.32670	5.32670	
EGD	12	6.21712	6.21712	6.21712
M11	12	6.98499 6.98499		
M14	12	7.14102 7.14102		
M1	12	7.41666		
Sig.		.371	.084	.472

**Thickness**

	N	Subset for alpha = .05			
STRAIN		1	2	3	4
M1	12	1.7119			
EGD	12	2.0045	2.0045		
M4	12	2.4074			2.4074
M14	12	2.7175			2.7175
M7	12	2.8971			2.8971
M11	12	3.0781			3.0781
Sig.		.708	.369	.172	.495

**Roughness**

	N	Subset for alpha = .05	
STRAIN		1	2
M14	12	.16216	
M11	12	.19513	
M7	12	.38207	
M1	12	.40937	
M4	12	.43433	
EGD	12	.49544	
Sig.		.988	.262

**Table 3.2-a. PCA results explaining total variance of extracted components**

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2.326	58.156	58.156	2.326	58.156	58.156
2	1.182	29.544	87.699	1.182	29.544	87.699
3	.417	10.427	98.126			
4	7.496E-02	1.874	100.000			

Extraction Method: Principal Component Analysis.

**Table 3.2-b. Component matrix showing values of variables associated with components 1 and 2 based on factor loadings**

	Component	
	1	2
<b>Biovolume</b>	<b>.945</b>	<b>.145</b>
<b>S/Bv Ratio</b>	<b>-.731</b>	<b>-.484</b>
<b>Thickness</b>	<b>-.256</b>	<b>.921</b>
<b>Roughness</b>	<b>.913</b>	<b>-.280</b>

Extraction Method: Principal Component Analysis - 2 components extracted.

**Table 3.3-a. Two-way ANOVA results of strain and temperature interaction on biofilm structures**

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
<b>Corrected Model</b>	Biovolume	7071301838.207	11	642845621.655	4.969	.000
	S/Bv Ratio	103.231	11	9.385	4.133	.000
	Thickness	19.453	11	1.768	7.588	.000
	Roughness	1.326	11	.121	8.510	.000
<b>Intercept</b>	Biovolume	20919448379.444	1	20919448379.444	161.715	.000
	S/Bv Ratio	2886.874	1	2886.874	1271.250	.000
	Thickness	439.059	1	439.059	1883.751	.000
	Roughness	8.640	1	8.640	609.834	.000
<b>STRAIN</b>	Biovolume	6127545873.811	5	1225509174.762	9.474	.000
	S/Bv Ratio	63.773	5	12.755	5.617	.000
	Thickness	16.905	5	3.381	14.506	.000
	Roughness	1.104	5	.221	15.586	.000
<b>TEMP</b>	Biovolume	311108329.674	1	311108329.674	2.405	.126
	S/Bv Ratio	3.061	1	3.061	1.348	.250
	Thickness	.607	1	.607	2.602	.112
	Roughness	1.015E-03	1	1.015E-03	.072	.790
<b>STRAIN * TEMP</b>	Biovolume	632647634.722	5	126529526.944	.978	.439
	S/Bv Ratio	36.396	5	7.279	3.205	.012
	Thickness	1.942	5	.388	1.666	.157
	Roughness	.221	5	4.423E-02	3.122	.014
<b>Error</b>	Biovolume	7761618893.169	60	129360314.886		
	S/Bv Ratio	136.254	60	2.271		
	Thickness	13.985	60	.233		
	Roughness	.850	60	1.417E-02		
<b>Total</b>	Biovolume	35752369110.820	72			
	S/Bv Ratio	3126.358	72			
	Thickness	472.497	72			
	Roughness	10.817	72			
<b>Corrected Total</b>	Biovolume	14832920731.376	71			
	S/Bv Ratio	239.484	71			
	Thickness	33.438	71			
	Roughness	2.176	71			

**Table 3.3-b. Two-way ANOVA results of temperature and shear force interaction on biofilm structures**

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
<b>Corrected Model</b>	Biovolume	2572682651.498	3	857560883.833	4.756	.005
	S/Bv Ratio	16.542	3	5.514	1.682	.179
	Thickness	.803	3	.268	.558	.645
	Roughness	.390	3	.130	4.948	.004
<b>Intercept</b>	Biovolume	20919448379.444	1	20919448379.444	116.027	.000
	S/Bv Ratio	2886.874	1	2886.874	880.530	.000
	Thickness	439.059	1	439.059	914.842	.000
	Roughness	8.640	1	8.640	328.897	.000
<b>SHEAR FORCE</b>	Biovolume	1182445232.882	1	1182445232.882	6.558	.013
	S/Bv Ratio	2.545	1	2.545	.776	.381
	Thickness	1.279E-02	1	1.279E-02	.027	.871
	Roughness	.220	1	.220	8.384	.005
<b>TEMP</b>	Biovolume	311108329.674	1	311108329.674	1.726	.193
	S/Bv Ratio	3.061	1	3.061	.934	.337
	Thickness	.607	1	.607	1.264	.265
	Roughness	1.015E-03	1	1.015E-03	.039	.845
<b>SHEAR * TEMP</b>	Biovolume	1079129088.942	1	1079129088.942	5.985	.017
	S/Bv Ratio	10.936	1	10.936	3.336	.072
	Thickness	.183	1	.183	.382	.538
	Roughness	.169	1	.169	6.422	.014
<b>Error</b>	Biovolume	12260238079.878	68	180297618.822		
	S/Bv Ratio	222.942	68	3.279		
	Thickness	32.635	68	.480		
	Roughness	1.786	68	2.627E-02		
<b>Total</b>	Biovolume	35752369110.820	72			
	S/Bv Ratio	3126.358	72			
	Thickness	472.497	72			
	Roughness	10.817	72			
<b>Corrected Total</b>	Biovolume	14832920731.376	71			
	S/Bv Ratio	239.484	71			
	Thickness	33.438	71			
	Roughness	2.176	71			



**Table 3.3-c. Two-way ANOVA results of strain and shear force interaction on biofilm structures**

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
<b>Corrected Model</b>	Biovolume	8603050085.507	11	782095462.319	7.532	.000
	S/Bv Ratio	99.818	11	9.074	3.898	.000
	Thickness	18.537	11	1.685	6.785	.000
	Roughness	1.527	11	.139	12.819	.000
<b>Intercept</b>	Biovolume	20919448379.444	1	20919448379.444	201.476	.000
	S/Bv Ratio	2886.874	1	2886.874	1240.182	.000
	Thickness	439.059	1	439.059	1767.904	.000
	Roughness	8.640	1	8.640	798.029	.000
<b>STRAIN</b>	Biovolume	6127545873.811	5	1225509174.762	11.803	.000
	S/Bv Ratio	63.773	5	12.755	5.479	.000
	Thickness	16.905	5	3.381	13.614	.000
	Roughness	1.104	5	.221	20.396	.000
<b>SHEAR FORCE</b>	Biovolume	1182445232.882	1	1182445232.882	11.388	.001
	S/Bv Ratio	2.545	1	2.545	1.093	.300
	Thickness	1.279E-02	1	1.279E-02	.052	.821
	Roughness	.220	1	.220	20.342	.000
<b>STRAIN * SHEAR</b>	Biovolume	1293058978.814	5	258611795.763	2.491	.041
	S/Bv Ratio	33.500	5	6.700	2.878	.021
	Thickness	1.619	5	.324	1.304	.274
	Roughness	.202	5	4.048E-02	3.738	.005
<b>Error</b>	Biovolume	6229870645.869	60	103831177.431		
	S/Bv Ratio	139.667	60	2.328		
	Thickness	14.901	60	.248		
	Roughness	.650	60	1.083E-02		
<b>Total</b>	Biovolume	35752369110.820	72			
	S/Bv Ratio	3126.358	72			
	Thickness	472.497	72			
	Roughness	10.817	72			
<b>Corrected Total</b>	Biovolume	14832920731.376	71			
	S/Bv Ratio	239.484	71			
	Thickness	33.438	71			
	Roughness	2.176	71			

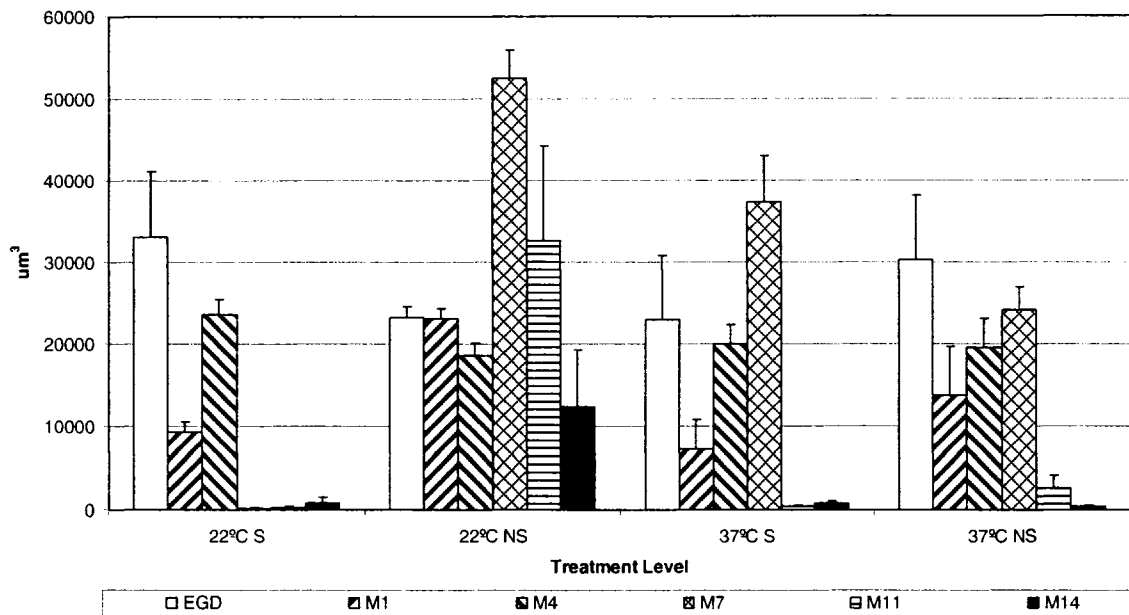


Fig. 3.1-a. Average biovolume & st. dev. of surface associated *L. monocytogenes* EGD & its corresponding mutant strains at 22°C and 37°C, with (S) and without shear force (NS)

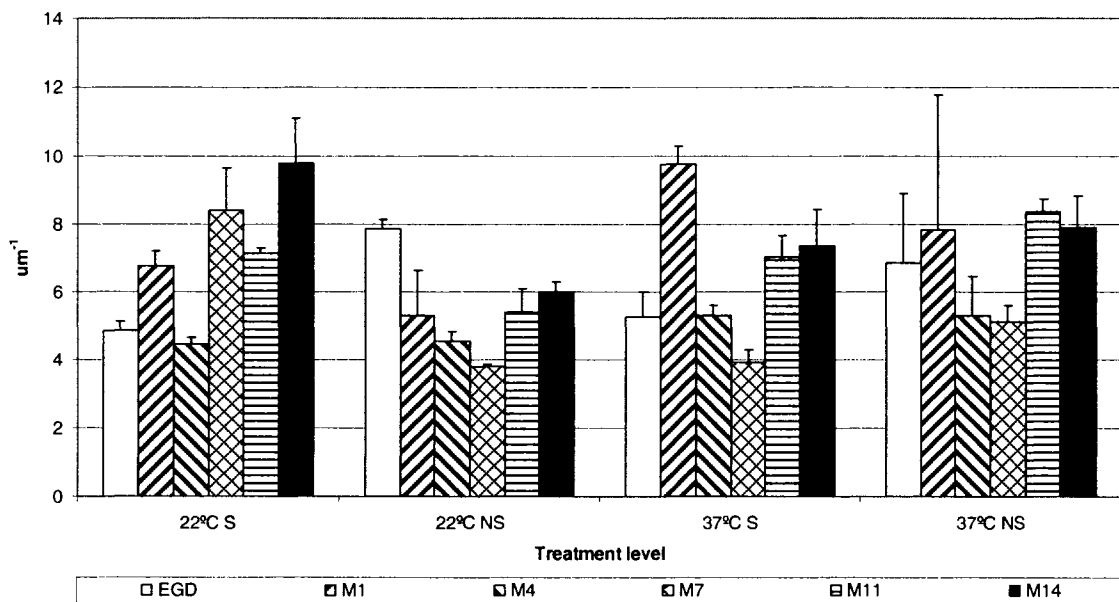


Fig. 3.1-b. Average surface area to biovolume ratio and st. dev. of surface associated *L. monocytogenes* EGD & its corresponding mutant strains at 22°C and 37°C, with (S) and without shear force (NS)

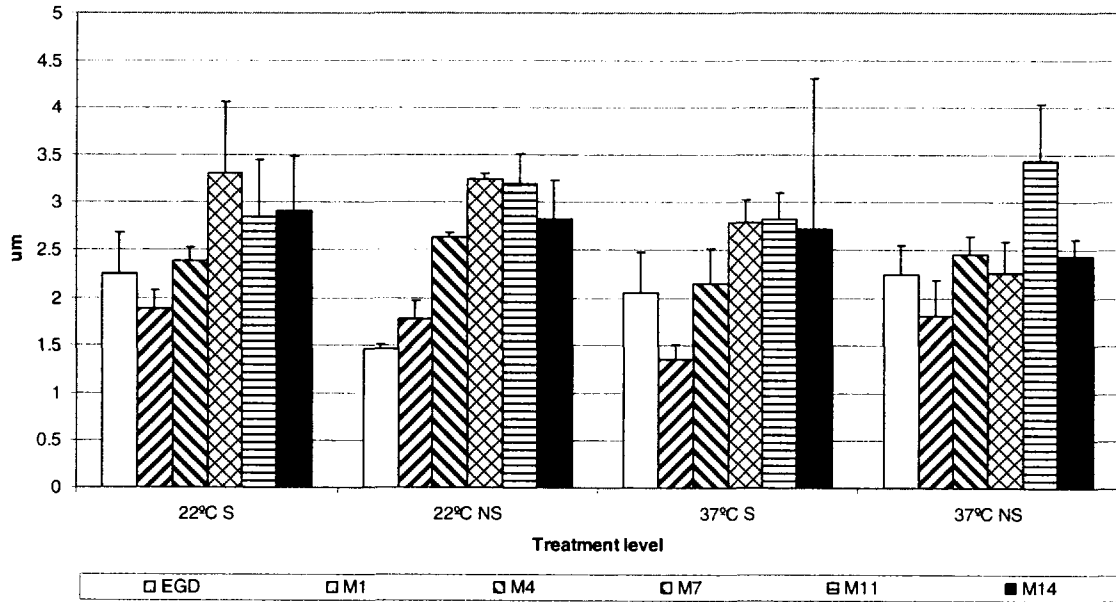


Fig. 3.1-c. Average thickness & st. dev. of surface associated *L. monocytogenes* EGD & its corresponding mutant strains at 22°C and 37°C, with (S) and without shear force (NS)

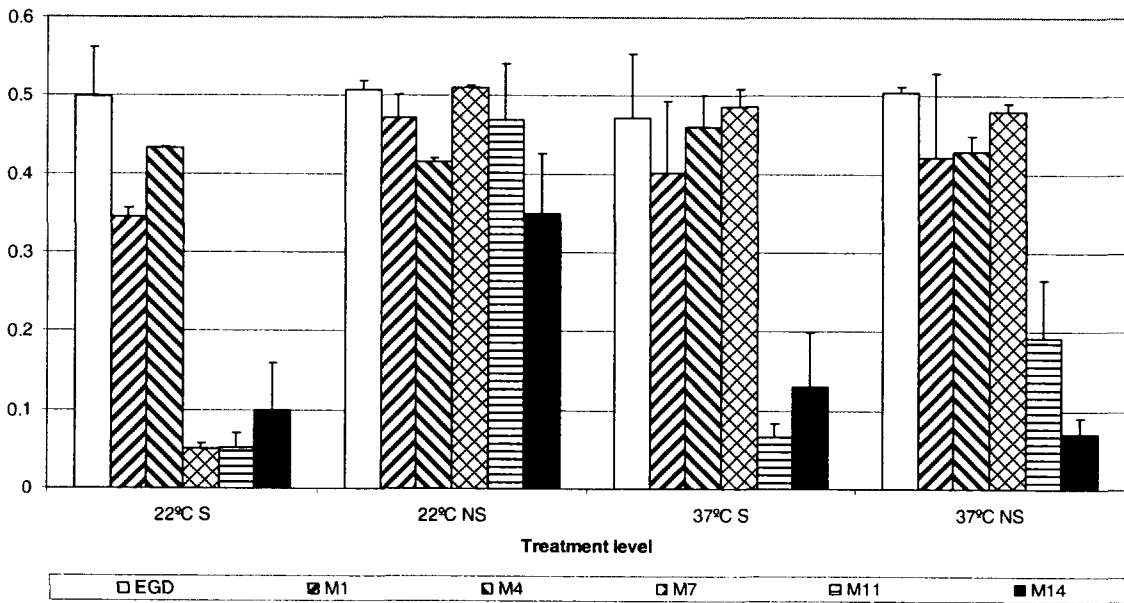


Fig. 3.1-d. Average roughness & st. dev. of surface associated *L. monocytogenes* EGD & its corresponding mutant strains at 22°C and 37°C, with (S) and without shear force (NS)

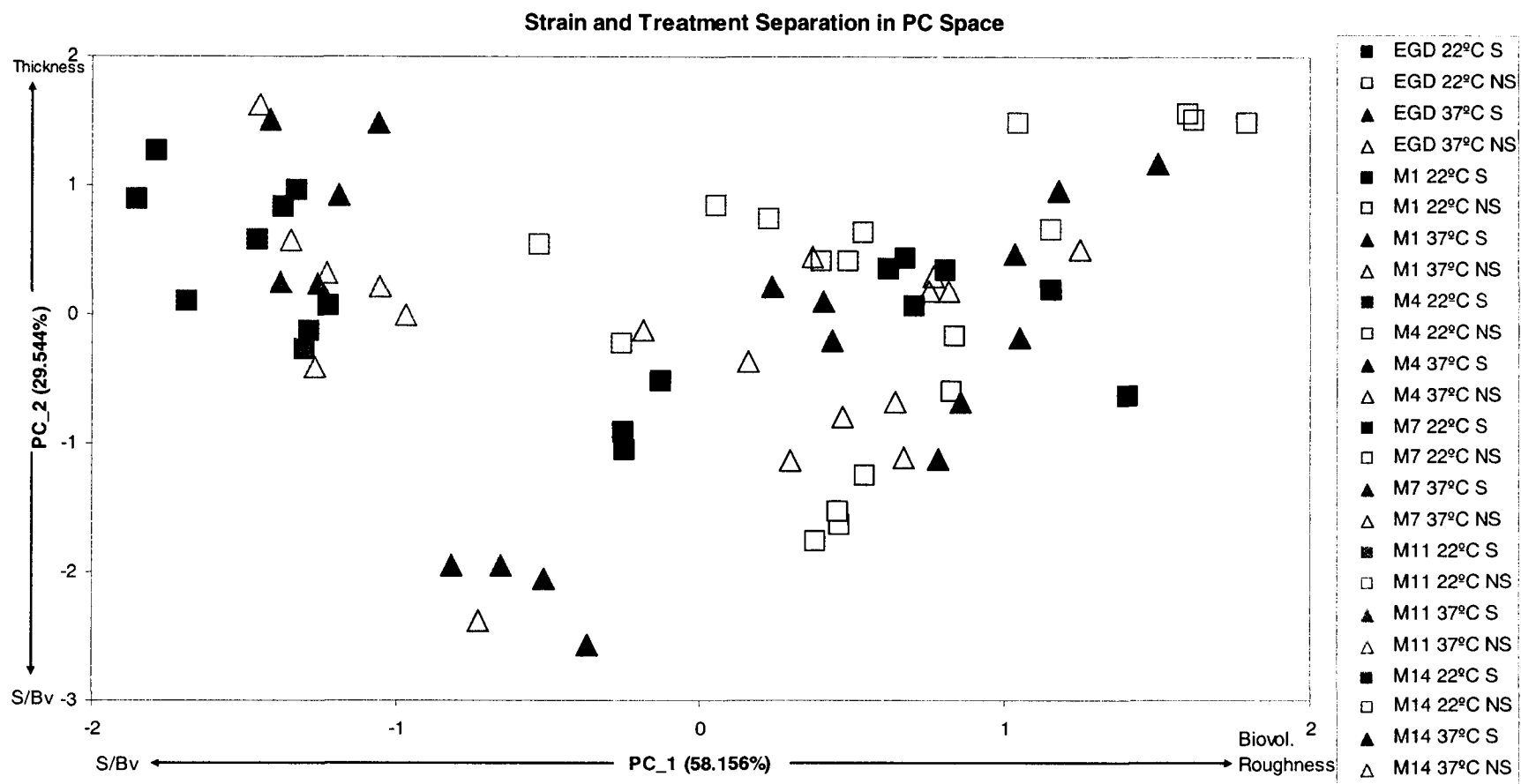


Fig. 3.2. Score plot of principal component analysis showing separation of *L. monocytogenes* wild-type EGD and mutant biofilms grown on glass at 4 different treatment levels

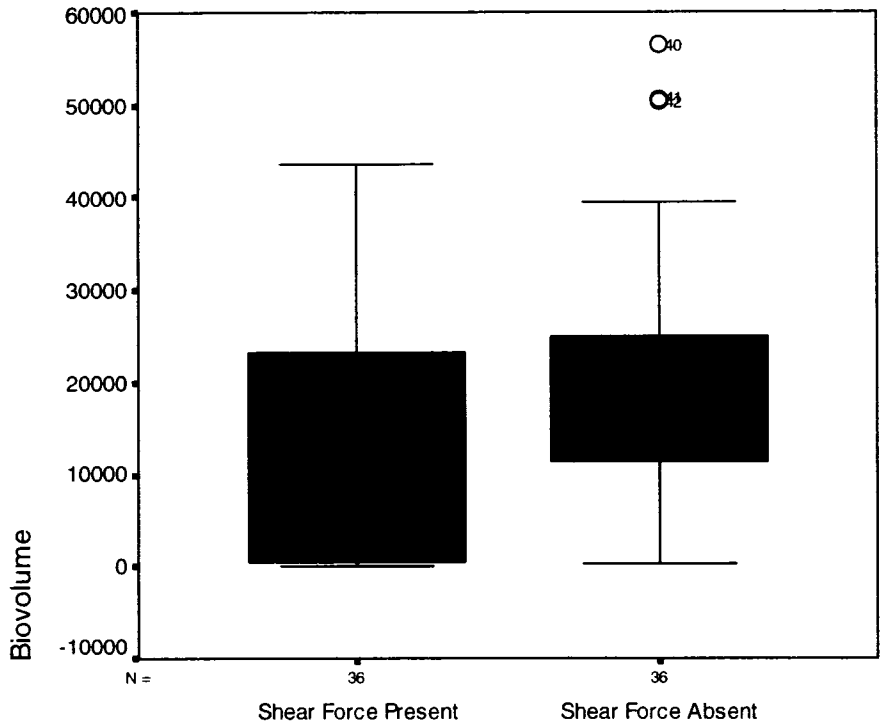


Fig. 3.3-a. Box plot exploring the effect of shear force on mean biofilm biovolume

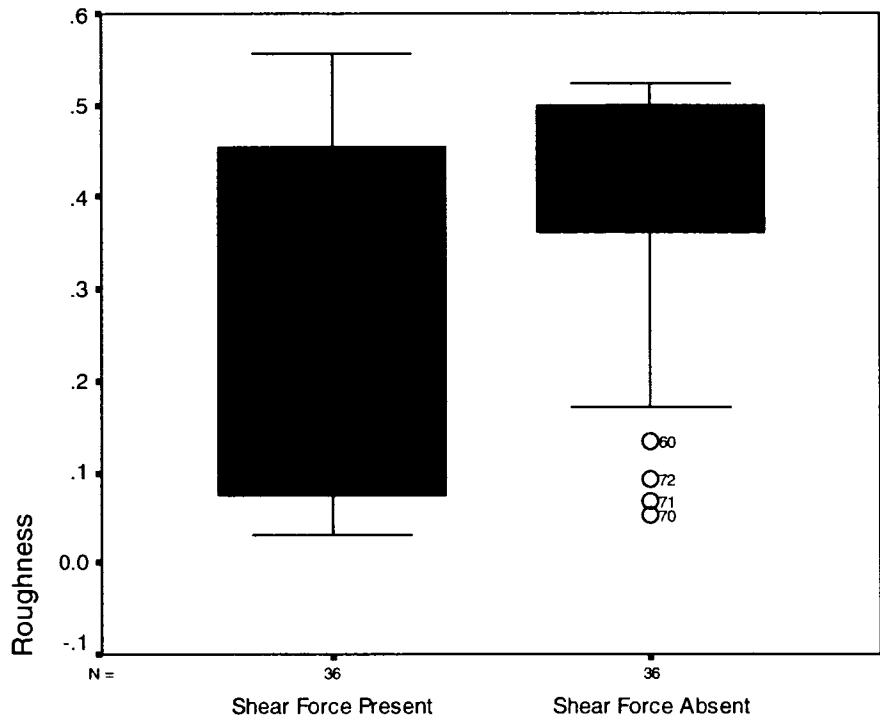


Fig. 3.3-b. Box plot exploring the effect of shear force on mean biofilm roughness

## References

- Allan, E., P. Mullany, and Tabaqchali, S. 1998. Construction and characterization of a *Helicobacter pylori clpB* mutant and role of the gene in the stress response. *J. Bacteriol.* 180(2):426-429.
- Baca-DeLancey, R. R., M. M. T. South, X. Ding, and Rather, P. N. 1999. *Escherichia coli* genes regulated by cell-to-cell signaling. *Proc. Natl. Acad. Sci. USA* 96:4610-4614
- Corona-Izquierdo, F. P. and Membrillo-Hernandez, J. 2002. A mutation in *rpoS* enhances biofilm formation in *Escherichia coli* during exponential phase of growth. *FEMS Microbiol. Lett.* 211:105-110.
- Carpentier, B., A. C. L. Wong, and Cerf, O. 1998. Biofilms on dairy plant surfaces what's new? *Bull. Int. Dairy Fed.* 329:32-35.
- Carpentier, B. and Cerf, O. 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Microbiology* 75:499-511.
- Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and Lappin-Scott, H. M. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* 49:711-445.
- DeBeer, D. G., P. Stoodley, F. L. Roe, and Lewandowski, Z. 1994 Effects of biofilm structures on oxygen distribution and mass transport. *Biotech. Bioeng.* 43:1131-1138.
- Digby, P. G. N. and Kempton, R. A. 1987. *Multivariate Analysis of Ecological Communities*, Chapman and Hall, New York.
- Eriksson, M. J. and Clarke, A. K. 1996. The heat shock protein ClpB mediates the development of thermotolerance in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J. Bacteriol.* 178(16):4839-4846.
- Flanary, P. L., R. D. Allen, L. Dons, and Kathariou, S. 1999. Insertional inactivation of the *Listeria monocytogenes cheYA* operon abolishes response to oxygen gradients and reduces the number of flagella. *Can. J. Microbiol.* 45:646-652.
- Goloubinoff, P., A. Mogk, A. P. Zvi, T. Tomoyasu, and Bukau, B. 1999. Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proc. Natl. Acad. Sci. USA* 96:13732-13737.
- Harris, R. J. 1985. *A Primer of Multivariate Statistics*, 2<sup>nd</sup> ed. Academic, New York.
- Hengge-Aronis R. 2002. Signal transduction and regulatory mechanisms involved in control of the  $\sigma^S$  (*RpoS*) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* 66:373-395.

Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. Ersboll, and Molin, S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146:2395-2407.

Hong, S. W. and Vierling, E. 2000. Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. *Proc. Natl. Acad. Sci.* 97(8):4392-4397.

Johnson, R. A. and Wichern, D. W. 1988. *Applied Multivariate Statistical Analysis*, Prentice Hall, New Jersey.

Kruger, E., U. Volker, and Hecker, M. 1994. Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. *J. Bacteriol.* 176:3360–3367.

Liu, S., J. E. Graham, L. Bigelow, P. D. Morse, and Wilkinson, B. J. 2002. Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. *Appl Environ. Microbiol.* 68(4):1697–1705.

Milohanic E., Pron B., Berche P., and Gaillard J.-L. 2000. Identification of new loci involved in adhesion of *Listeria monocytogenes* to eukaryotic cells. *Microbiology* 146: 731-739.

Mogk, A., T. Tomoyasu, P. Goloubinoff, S. Rudiger, D. Roder, H. Langen, and Bukau, B. 1999. Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO J.* 18:6934–6949.

Mueller, L. N., J. F. C. de Brouwer, J. B. Xavier, and Almeida, J. S. (2004), “PHLIP: Image bioinformatics for the quantitative analysis of confocal laser scanning microscopy data”, *Proceedings IWA Biofilm symposium*. Las Vegas, Nevada.

Nandakumar, K., W. Keeler, H. Schraft, and Leung, K. T. 2006. Visible laser and UV-A radiation impact on a PNP degrading *Moraxella* strain and its *rpoS* mutant. *Biotechnol. Bioeng.* 94(4):793-802.

Neff, W. A. and Marcus, L. F. 1980. *A survey of Multivariate Methods for Systematics*, Am. Mus. Nat. Hist., New York.

Neuwald, A. F., L. Aravind, J. L. Spouge, and Koonin, E. V. 1999. AAA<sup>+</sup>: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* 9(1):27-43.

Pielou, E. C. 1984. *The interpretation of Ecological Data: A Primer on Classification and Ordination*, Wiley, New York.

- Polard, P., S. Marsin, S. McGovern, S. D. Ehrlich and Bruand, C. 2002. Functional characterization of the primosome protein *PriA* of *Bacillus subtilis*. Nucl. Acids Res. 30: 1593-1605.
- Porankiewicz, J. and A. K. Clarke. 1997. Induction of the heat shock protein ClpB affects cold acclimation in the cyanobacterium *Synechococcus* sp. strain PCC 7942. J. Bacteriol. 179:5111-5117.
- Pratt, L. A. and Kolter, R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis, and type I pili. Molec. Microbiol. 30(2):285-293.
- Ronner, A. B. and Wong, A. C. L. 1993. Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-N rubber. J. Food Prot. 56:750-758.
- Rouquette, C., M. T. Ripio, E. Pellegrini, J. M. Bolla, R. I. Tascon, J. A. Vazquez-Boland, and Berche, P. 1996. Identification of a ClpC ATPase required for stress tolerance and *in vivo* survival of *Listeria monocytogenes*. Mol. Microbiol. 21:977-987.
- Sagripanti, J. L. and Bonifacino, A. 2000. Resistance of *Pseudomonas aeruginosa* to liquid disinfectants on contaminated surfaces before formation of biofilms. J. AOAC Int. 83:1415-1422.
- Sanchez, Y. and Lindquist, S. L. 1990. HSP104 required for induced thermotolerance. *Science* 248:1112-1115.
- Squires, C. and Squires, C. L. 1992. The Clp proteins: proteolysis regulators or molecular chaperones? J. Bacteriol. 174:1081-1085.
- Squires, C. L., S. Pedersen, B. M. Ross, and Squires, C. 1991. ClpB is the *Escherichia coli* heat shock protein F84.1. J. Bacteriol. 173:4254-4262.
- Sutherland, I. W. 2001. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147:3-9.
- Van der Ploeg, J. R., E. Eichhorn, and Leisinger, T. 2001. Sulfonate-sulfur metabolism and its regulation in *Escherichia coli*. Arch. Microbiol. 176(1-2):1-8.
- Zolkiewski, M. 1999. ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. J. Biol. Chem. 274:28083-28086.



## Chapter Four

### Complementation *in Trans* of a *Listeria monocytogenes* EGD *clpB* Mutant Defective in Biofilm Development

#### 4.1. INTRODUCTION

*Listeria monocytogenes* is a gram-positive pathogen that causes listeriosis – an illness with a high degree of mortality for immunocompromised hosts. During the past few years, this bacterium has been extensively studied because of its ability to grow and form biofilms on food stored at refrigeration temperatures. Contamination occurs, which poses a significant public health threat, leading to costly product recalls in the food industry (Mead *et al.* 1999).

In response to stress conditions, bacteria transiently increase the expression of genes for stress response proteins which are thought to protect the cell from stress-induced damage by preventing denaturation of cellular proteins, reactivating once-inactivated proteins, and regulating the degradation of irreversibly denatured proteins (Parsell and Lindquist 1993). Studies of the immune response to stress proteins have demonstrated that they are major antigens of many bacterial pathogens (Dunn *et al.* 1992; Kaufmann and Schoel 1994; Suerbaum *et al.* 1994), suggesting that they are abundant in the bacterial cell during infection. The Clp ATPase protein family, which comprises ClpA-, ClpB-, ClpC-, and ClpX-like proteins, includes several stress response proteins (Squires and Squires 1992). These caseinolytic proteins (Clps) form large protein complexes that have both proteolytic and chaperone activities (Gottesman and Maurizi 1992) which are involved in the degradation of damaged polypeptides and the salvage of amino acids. Conditions which induce expression of the Clp stress proteins include high temperature, high salt or ethanol concentration, oxygen limitation, and iron limitation.

Three *clp* genes of *L. monocytogenes* have been shown to be controlled by the CtsR repressor of stress response genes (Nair *et al.* 2000), and many *clpB* genes are known to be heat shock-induced. In a study by Chastanet *et al.* (2004), ClpB was found

to have no obvious role in terms of stress tolerance, but is required for induced thermotolerance of *L. monocytogenes*. Allan *et al.* (1998) determined the role of the *clpB* gene product in *H. pylori* by constructing an isogenic *clpB* mutant, and comparing it with the parental strain for survival at high temperature. In their study, the *clpB* mutant showed increased sensitivity to high-temperature stress, indicating that the *clpB* gene product is a stress response protein which may be important for survival of *H. pylori* in the hostile environment of the human stomach. While investigating into stress tolerance, intracellular replication and biofilm formation in *Staphylococcus aureus*, Frees *et al.* (2004) found that the absence of *clpB* only influenced tolerance to heat while leaving tolerance to other stresses and synthesis of virulence factors unaffected. In addition, they also found that biofilm formation was reduced in the absence of particular *Clps*. ClpB induction was also shown to play a critical role in cold acclimation in the cyanobacterium *Synechococcus* sp. strain PCC7942 (Porankiewicz and Clarke 1997). ClpB mutants therefore present themselves as attractive model strains for examining the significance of thermotolerance or cold acclimation in biofilm production.

To elucidate the role of *clpB* regarding biofilm production and formation, we attempted restoring a *L. monocytogenes clpB* mutant to its wild-type parent strain through complementation *in trans*. Previously, a *L. monocytogenes clpB* mutant M11 was tested for its ability to form biofilms on microtitre plates. It was found that this mutant had a reduced ability to adhere to a polystyrene surface after being stained with crystal violet (Chae 2004). Our other studies involving planktonic growth and biofilm production of *L. monocytogenes* EGD on glass also showed reduced planktonic growth rates for this mutant at 22°C, and reduced biofilm production at 37°C without shear force, when compared to its wild-type parent. In addition, mutant M11 was also observed forming biofilms with high thickness and surface to biovolume ratio with scanning electron microscopy (SEM). Thus, by restoring the *clpB* gene to mutant M11, we can ascertain that the phenomenon seen in our studies with *L. monocytogenes* biofilms regarding growth and production on glass can be attributed to the *clpB* gene being absent.

The objectives of this study were to design primers to target the *clpB* gene of wild-type *L. monocytogenes* EGD, amplify the *clpB* fragment using polymerase chain reaction (PCR), clone the amplified fragment into the pGEM-T easy vector, subclone this

same fragment into the pMK4 shuttle vector, transform the construct containing the full-length *clpB* gene into *E. coli* JM109, and subsequently into *L. monocytogenes* EGD for complementation *in trans*.

## **4.2. MATERIALS AND METHODS**

### **4.2.1. Bacterial Strains and Plasmids**

Cloning of the *clpB* gene from *L. monocytogenes* EGD was carried out to achieve functional complementation of the *L. monocytogenes* mutant strain M11. The *E. coli* strain JM109 was used as a host for recombinants made by plasmids pGEM-T and pMK4. The *L. monocytogenes* mutant M11 was generated and genetically characterized by Chae (2004). Plasmids pGEM-T and pMK4 were obtained from Promega and the National Collections of Industrial Food and Marine Bacteria (NCIMB) respectively. The pMK4 shuttle vector carries chloramphenicol and ampicillin (expressed in *E. coli*) resistance markers (Fig. 4.1).

### **4.2.2. Culture Conditions**

*L. monocytogenes* and *E. coli* cultures were grown at 37°C with shaking. The growth medium for *L. monocytogenes* was trypticase soy broth (TSB; Difco Laboratories, Detroit, Michigan, USA), and Luria-Bertani broth (LB; Difco Laboratories, Detroit, Michigan, USA) was used for *E. coli*.

### **4.2.3. Preparation of Genomic DNA**

Harvested *L. monocytogenes* EGD cells (50 mL) were lysed by treatment with lysozyme (10 mg/mL) in 50mM EDTA at 37°C for 60 min. Genomic DNA was prepared

by using the Wizard Genomic DNA Purification Kit as described by the manufacturer (Promega, Madison, Wis.). Chromosomal DNA concentration was determined by UV spectroscopy at 260 nm.

#### 4.2.4. Molecular Cloning Strategy

An overview of the molecular cloning strategy is shown in Figure 4.2.

##### 4.2.4.1. Cloning of *clpB* into pGEM-T Easy Vector

The published sequence for *L. monocytogenes clpB* (GenBank accession no. CAD00284) was used to design primers for the amplification of the entire *clpB* gene with total flanking sequence of 406 bp on both ends. Primers were designed with reference to the nucleotide sequence lmo2206 (*clpB*) in GenBank. The *clpB* gene was located from position 2294555 to 2297155 (2600bp) on the *L. monocytogenes* EGD genome. The forward primer (ClpB-F) was designed 174bp upstream (position 2294381 on the *L. monocytogenes* EGD genome) from the *clpB* gene to include the potential -35 and -10 promoter, transcriptional start site and RBS sequence (Fig. 4.3). The reverse primer (ClpB-R) on the other hand, was designed 232bp downstream (position 2297387 on the *L. monocytogenes* EGD genome) from the *clpB* gene. Primer selection was based on melting temperature ( $T_m$ ) which was determined by DNAMan for Windows (Lynnon Corporation, Quebec, Canada). From primer analysis, melting temperatures for the sense and antisense primers were 62.3 and 62.5°C respectively. Thirty-mer oligonucleotide pairs Clpb-F (5'-CGCGGGATCCGTACCAATGCTTGCGCTGAA-3'), incorporating the underlined site for *Bam*HI) and Clpb-R (5'-CGCGGGATCCCGACAAGTCCACATCATGCC-3'), incorporating the underlined site for *Bam*HI) were then designed and synthesized by Sigma Genosys. PCR amplification from wild-type chromosomal DNA was carried out in a 50  $\mu$ L volume reaction using 50 ng of *L. monocytogenes* genomic DNA in the presence of 2mM MgCl<sub>2</sub>, 1 $\mu$ M of each PCR primer, 0.2mM dNTPs, 1x PCR reaction buffer and 5U of Taq DNA polymerase

using a PCR Sprint thermal cycler (Hybaid, Ashford, Middlesex). PCR conditions were set as follows. After initial denaturation for 10 min at 94°C, 30 cycles of amplification consisting of denaturation for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 3 min at 72°C were performed. This procedure was then followed by a final extension of 10 min at 72°C. The 3026-bp amplified PCR product was analyzed by agarose gel electrophoresis, quantified using UV spectroscopy at 260 nm, and cloned into the pGEM-T Easy Vector using the pGEM-T and pGEM-T Easy Vector Systems (Promega, Madison, Wis.) according to the manufacturer's instructions. Clones were selected on LB agar containing ampicillin (100 µg/ml) supplemented with IPTG and X-Gal. Suspected colonies of *E. coli* JM109 containing recombinant plasmids were replica plated and then grown overnight at 37°C in 50 mL LB broth supplemented with ampicillin. These cultures were subjected to a plasmid extraction with Promega's Wizard Plus SV Minipreps DNA Purification System, followed by agarose gel electrophoresis. Identification of products was done by cleaving the plasmids of suspected isolates with *Bam*HI and *Sal*I. To confirm presence of the *clpB* gene, recombinant plasmids showing presence of inserts by agarose gel electrophoresis were sequenced with primers T7 and SP6 at the Institute for Molecular Biology and Biotechnology Laboratory (MobixLab) at McMaster University. Each sequence obtained was compared and aligned with those in GenBank by using the BLASTX program (Altschul *et al.*, 1990).

#### 4.2.4.2. Subcloning of *clpB* into pMK4 Shuttle Vector

*Bacillus subtilis* containing the pMK4 shuttle vector was grown on LB medium containing chloramphenicol (50 µg/mL). *B. subtilis* cells (20 mL) were harvested by centrifugation and suspended in 1 mL of phosphate buffer saline (PBS). Cells were then lysed by treatment with lysozyme (10 mg/mL) in 50mM EDTA at 37°C for 60 min. In addition, *E. coli* cells containing the pGEM-T Easy Vector with *clpB* insert were grown on LB medium containing ampicillin (100 µg/mL) and 10 mL of cells were harvested for plasmid extraction. Plasmid DNA (pGEM-T and pMK4) was prepared by using a Wizard Plus SV Miniprep DNA Purification System as described by the manufacturer (Promega,

Madison, Wis.). A double digest involving *Bam*HI and *Eam*1105I was performed on plasmid pGEM-T to isolate the DNA fragment containing *clpB*. Cleaving of the pGEM-T plasmid was carried out in a 30  $\mu$ L volume reaction involving 20  $\mu$ L of template DNA in the presence of 3  $\mu$ L 10x reaction buffer, 4  $\mu$ L nuclease free water, 10 units enzyme *Bam*HI, and 20 units enzyme *Eam*1105I in a 0.2 mL PCR tube at 37°C for 1 hr. The pMK4 shuttle vector was restricted with of *Bam*HI to linearize the plasmid to allow for sticky end ligation. Cleaving of the pMK4 plasmid was carried out in a 35  $\mu$ L volume reaction involving 20  $\mu$ L of template DNA in the presence of 3.5  $\mu$ L 10x reaction buffer, 9.5  $\mu$ L nuclease free water, and 20 units enzyme *Bam*HI in a 0.2 mL PCR tube at 37°C for 1 hr. The desired cleaved DNA fragments were then isolated by agarose gel electrophoresis using TAE buffer and 0.7% low electroendosmosis agarose (Fermentas Top Vision LE GQ Agarose). Using longwave UV light for visualization of DNA, the bands corresponding to both insert (*clpB*) and vector (pMK4) were excised from the gel. Purification of DNA away from the gel material was performed using Promega's Wizard SV Gel and PCR Clean-up System as described by the manufacturer. Purified fragments were analyzed by agarose gel electrophoresis and quantified using the Genequant II RNA/DNA calculator (Hoefer Pharmacia Biotech, San Francisco, CA, USA). A dephosphorylation with calf intestine alkaline phosphatase (CIAP) was next performed for shuttle vector pMK4. Insert (261 ng) was then subcloned into pMK4 shuttle vector (180 ng) using T4 DNA ligase. The resulting product was then transformed using Fermentas' TransformAid Bacterial Transformation Kit as described by the manufacturer into *E. coli* JM109. Ap<sup>R</sup> Cm<sup>R</sup> resistant clones were selected on LB medium supplemented with ampicillin (50  $\mu$ g/ml), chloramphenicol (20  $\mu$ g/ml), IPTG and X-Gal.

### 4.3. RESULTS

#### 4.3.1. PCR Analysis of *ClpB*

Results from agarose gel electrophoresis of the PCR product revealed a band of approximately 3kb in size (Fig. 4.4). The positive control, which was a PCR

amplification of a 500-600bp section of the 16S rDNA gene revealed a band of the expected size with 16S universal 341-f and 907-r primers. Bands were absent for the negative control (template DNA replaced by ddH<sub>2</sub>O).

#### 4.3.2. Cloning of PCR Product into pGEM-T Easy Vector

In total, three isolates expected to contain positive clones were screened from white colony growth on LB media with ampicillin supplemented with IPTG and X-Gal. A restriction analysis with *Bam*HI and *Sal*I was performed for plasmids of isolates 1, 2 and 3 (Fig. 4.5, b, c, d, e, f, and g). A single digest with *Sal*I cuts the plasmid construct at location 90 of the vector which would reveal a 6041bp band size, whereas a digest with *Bam*HI would cut the construct at two locations which would result in two bands each of 3026bp (insert with flanking regions) and 3015bp (vector), when performed with agarose gel electrophoresis. From results, the cut with *Bam*HI revealed band sizes of approximately 3, and between 5 to 6kb for isolate 1; 6kb for isolate 2, and 3.5kb for isolate 3 (Fig. 4.5, b, d, and f). In addition, the cut with *Sal*I revealed band sizes of approximately 3.5kb and 6kb for isolate 1; 6kb for isolate 2, and between 6 to 8kb for isolate 3 (Fig. 4.5, c, e, and g).

Sequence analyses of plasmids from isolates 1 and 3 revealed the presence of the PCR product, and alignment analyses showed that the PCR product sequence matched that of *ClpB* in GenBank (data not shown).

#### 4.3.3. Subcloning of *ClpB* Insert into pMK4 Shuttle Vector

Agarose gel electrophoresis of the plasmid construct (pGEM-T Easy Vector with *clpB*) and pMK4 shuttle vector revealed approximate band sizes of 6kb and 5.6kb respectively (Fig. 4.6, b1, b2, c1, and c2). A double digest with *Bam*HI and *Eam*1105I was performed on the plasmid construct because of similar insert and vector sizes. *Eam*1105I cleaved the vector at location 1410, resulting in 2 fragments of 1.41kb and

1.605kb sizes; whereas *Bam*HI cleaved the construct at 2 locations resulting in a fragment of approximate 3kb size. This 3kb band size observed from agarose gel electrophoresis confirmed the presence of the *clpB* insert (Fig. 4.6, e). The shuttle vector pMK4 was linearized by cleaving with *Bam*HI to prepare it for the ligation procedure (Fig. 4.6, g).

The cleaved, purified linearized plasmid and insert were ligated together and agarose gel electrophoresis revealed a construct of approximately 9kb in size (Fig. 4.7, f) along with other undesired constructs with observed band sizes of approximately 6kb, 12kb and 15kb. The construct was then transformed into *E. coli* JM109. Results from the transformation procedure produced no positive clones on LB medium supplemented with ampicillin, chloramphenicol, IPTG and X-Gal. However, blue colony growth was observed for the positive control with supercoiled pMK4 plasmid. The negative control with ligated construct replaced by *E. coli* JM109 showed no growth on LB antibiotic plates with IPTG and X-Gal. Subsequent attempts at transformation still resulted in failure to obtain positive clones. In total, the ligation procedure along with transformation into *E. coli* JM109 was attempted five times with five different ligation products (Fig. 4.7, f and Fig. 4.8, b, d, f, and h).

#### 4.4. DISCUSSION

Our attempt at restoring a *clpB* mutant to its wild-type parent through complementation *in trans* was partially successful. We were able to amplify the *clpB* gene, clone it into the pGEM-T easy vector, and subsequently into the pMK4 shuttle vector. However, difficulty in transforming the plasmid construct into *E. coli* JM109 was faced after several attempts. Reasons for our inability to obtain positive clones may be attributed to several factors for e.g. pMK4 being a low copy number plasmid, low DNA yield for this plasmid, and spontaneous binding/joining of products (insert and vector) resulting in undesirable constructs during ligation.

Even though the pMK4 shuttle vector is a low copy number plasmid, we selected to use the pMK4 shuttle vector because, to restore mutant M11 to its wild-type parent strain, this plasmid would have to be placed into *Listeria*. The pMK4 shuttle vector was



selected because among broad host range shuttle plasmids replicating in *Escherichia coli* and *L. monocytogenes*, pMK4 has been shown to be most extensively used for complementation of mutants obtained by transposon insertion or for expression of gene products via their own promoters (Sullivan *et al.* 1984; Wirth *et al.* 1986; Cossart *et al.* 1989; Freitag 2000). Results from the positive control plates revealed blue colony growth with supercoiled plasmid, after transformation was performed. This meant that the pMK4 shuttle vector without insert did indeed transform into *E. coli* JM109. From DNA concentration estimates using the Fermentas 1kb DNA gene ruler, it was observed that the supercoiled pMK4 shuttle vector after extraction from *B. subtilis* contained about 920 ng of DNA compared to the *clpB* insert which was about 4 times higher in concentration. Because our procedure required several steps which involved cleaving with endonucleases, purification and dephosphorylation, DNA concentrations of plasmid pMK4, including the insert *clpB* were compromised at each subsequent stage, thus resulting in lower DNA yields than measured just after the plasmid extraction procedure from *B. subtilis* and *E. coli* JM109. This was observed in our ligation agarose gel electrophoresis results as bands showing the desired construct were faint. Thus, higher DNA yields with a higher copy number plasmid would have probably increased the probability of obtaining positive clones after transformation.

After ligation, several bands were observed on agarose gels run to check for desired product(s). These bands were a result of spontaneous or random binding involving insert and vector because both the pMK4 shuttle vector and *clpB* insert possessed *Bam*HI restriction cut sites. Thus, inserts could ultimately bind to one another, and similarly for vectors; two inserts could also bind to one vector, two vectors to one insert, and single vector to insert; which would result in band sizes of approximately 6kb, 12kb, 12kb, 15kb and 9kb respectively. This uncontrolled phenomenon may have affected DNA yield of desired ligated products which in turn, affected transformation into *E. coli* JM109. Thus, purification of DNA from the desired 9kb band away from the gel material could have been further performed to separate undesirable ligation products from the desired one.

Lastly, an attempt at using electroporation in place of transformation could have been performed. Our study involved using *E. coli* JM109 as an initial host for the plasmid

construct containing the pMK4 shuttle vector and *clpB* insert. As *Listeriae* are not naturally competent, although competence genes have been found in *L. monocytogenes* genome (Glaser *et al.* 2001), electroporation of the desired plasmid construct into *E. coli* JM109 would have made it easier for subsequent transformation of plasmid DNA into *L. monocytogenes* mutant M11 by electroporation (Vicente *et al.* 1987; Luchansky *et al.* 1988). Although this was not performed, electroporation along with other improvements to the complementation protocol suggested above should increase the probability of obtaining positive clones.

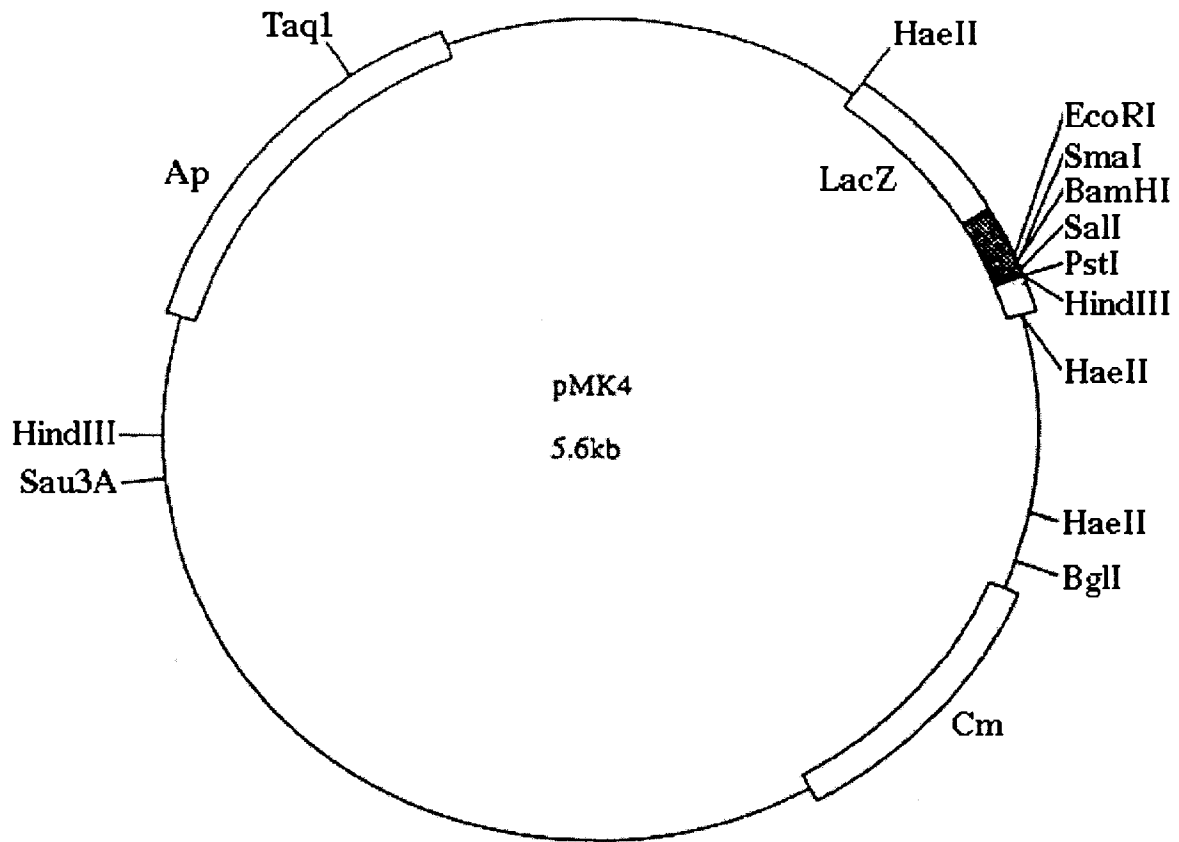


Fig. 4.1. Physical map of the pMK4 plasmid. The ampicillin (Ap) and chloramphenicol (Cm) resistance genes allow selection in *E. coli* and *L. monocytogenes*, respectively. From Sullivan *et al.* (1984)

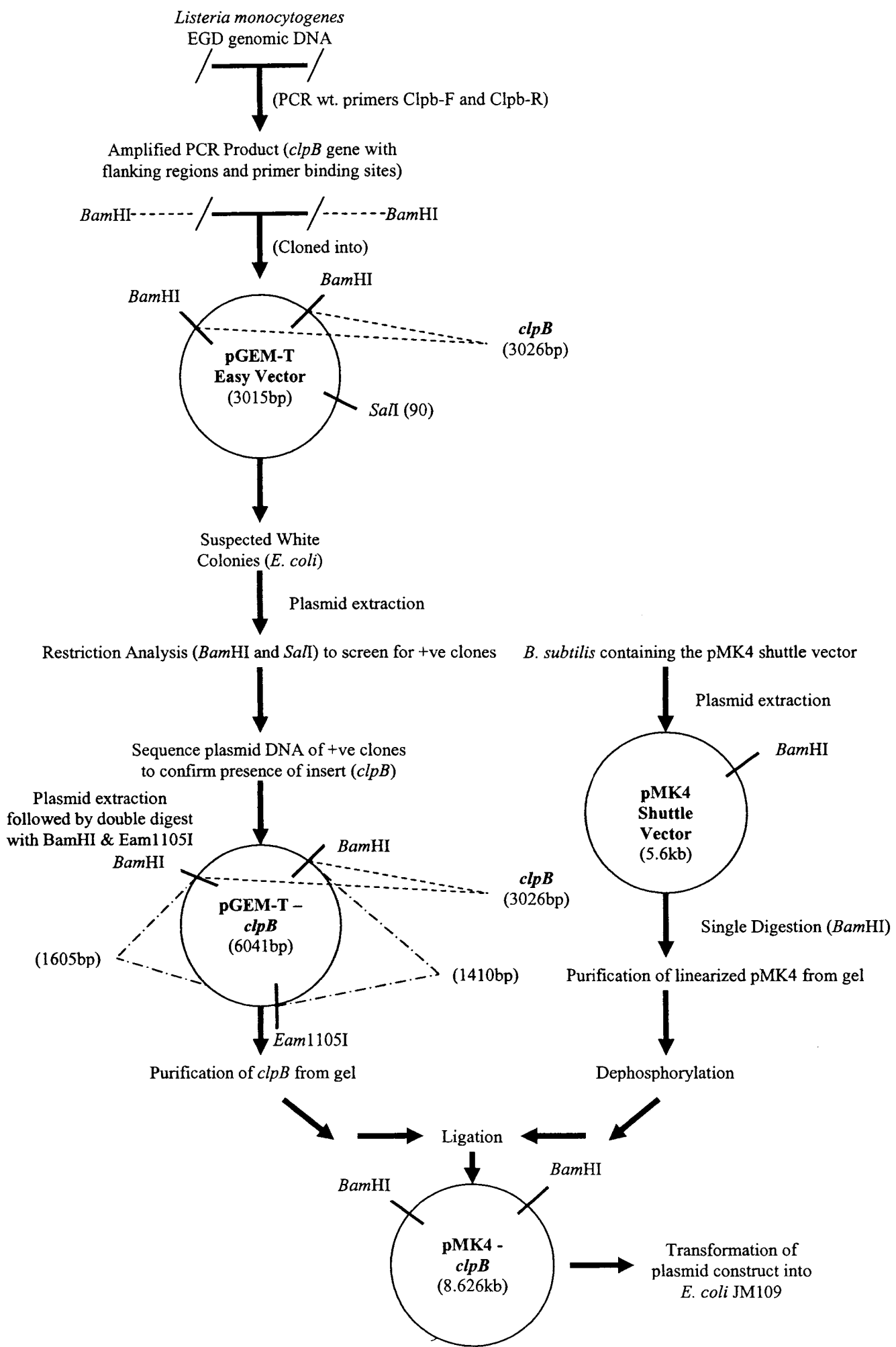


Fig. 4.2. Molecular cloning strategy.

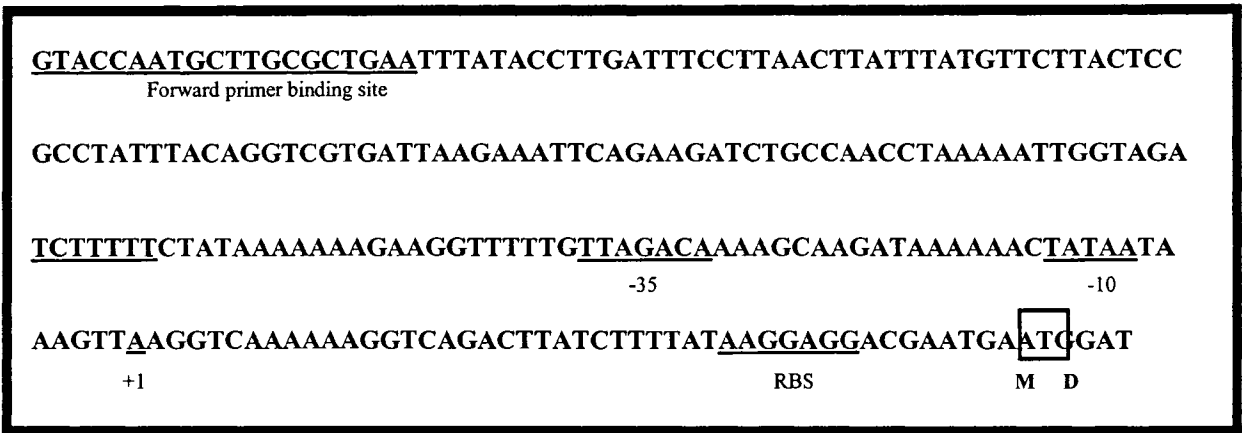


Fig. 4.3. Nucleotide sequence of the *L. monocytogenes* EGD *clpB* promoter region and primer binding site. Potential -35 and -10 promoter sequences are underlined; the transcriptional start site is indicated by +1; the potential RBS sequence is underlined and the translational start site is boxed.

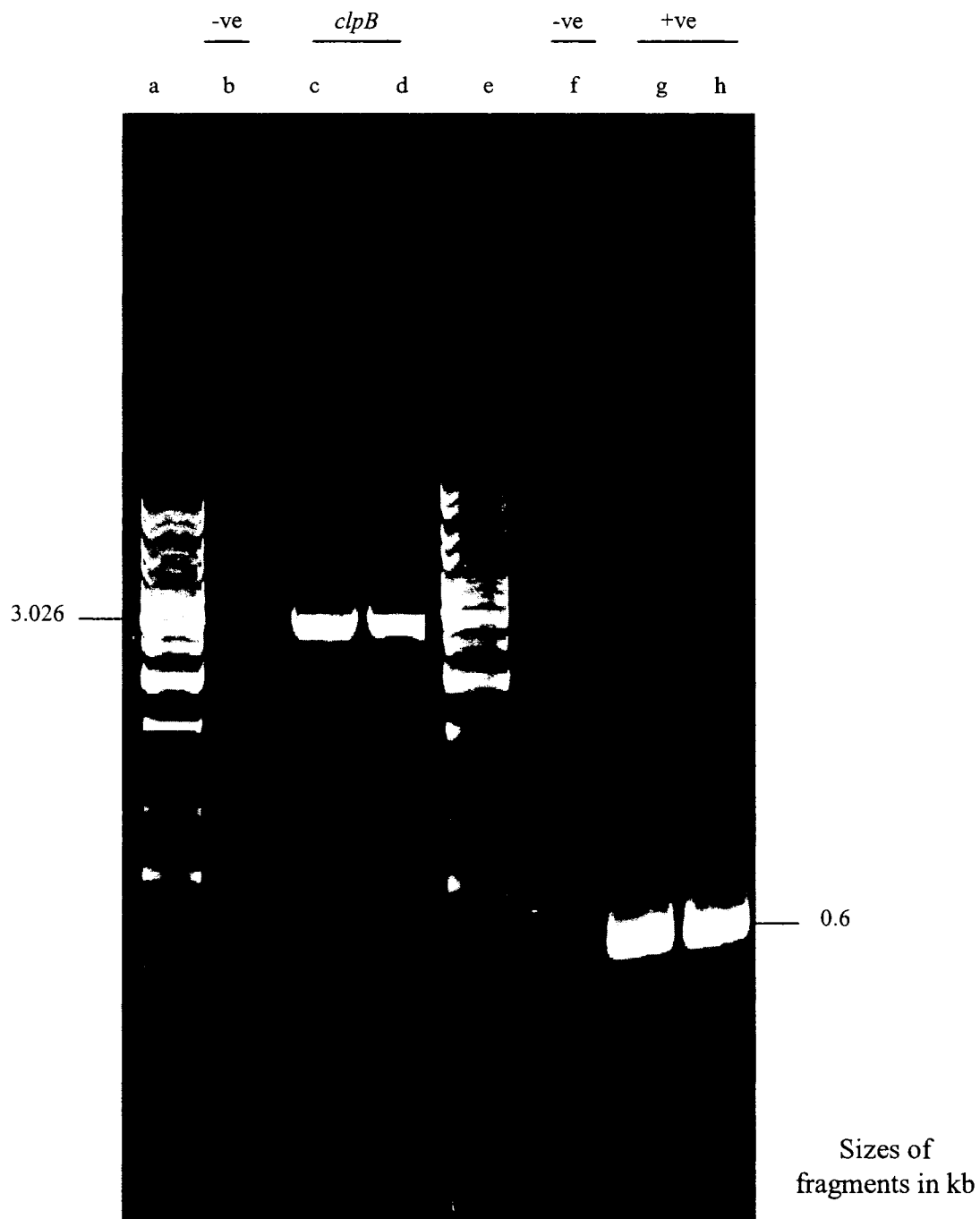


Fig. 4.4. PCR analysis of the *L. monocytogenes clpB* gene. Both (c) and (d) are PCR products from the primer extension of *clpB*; (a) and (e) are DNA ladders (1kb), (b) and (f) are negative controls (template DNA replaced with water) obtained from PCR with rest-*clpB* and 16-sr universal primers respectively, (g) and (h) are positive controls (template DNA similar to c and d) obtained from PCR with 16-sr universal primers.

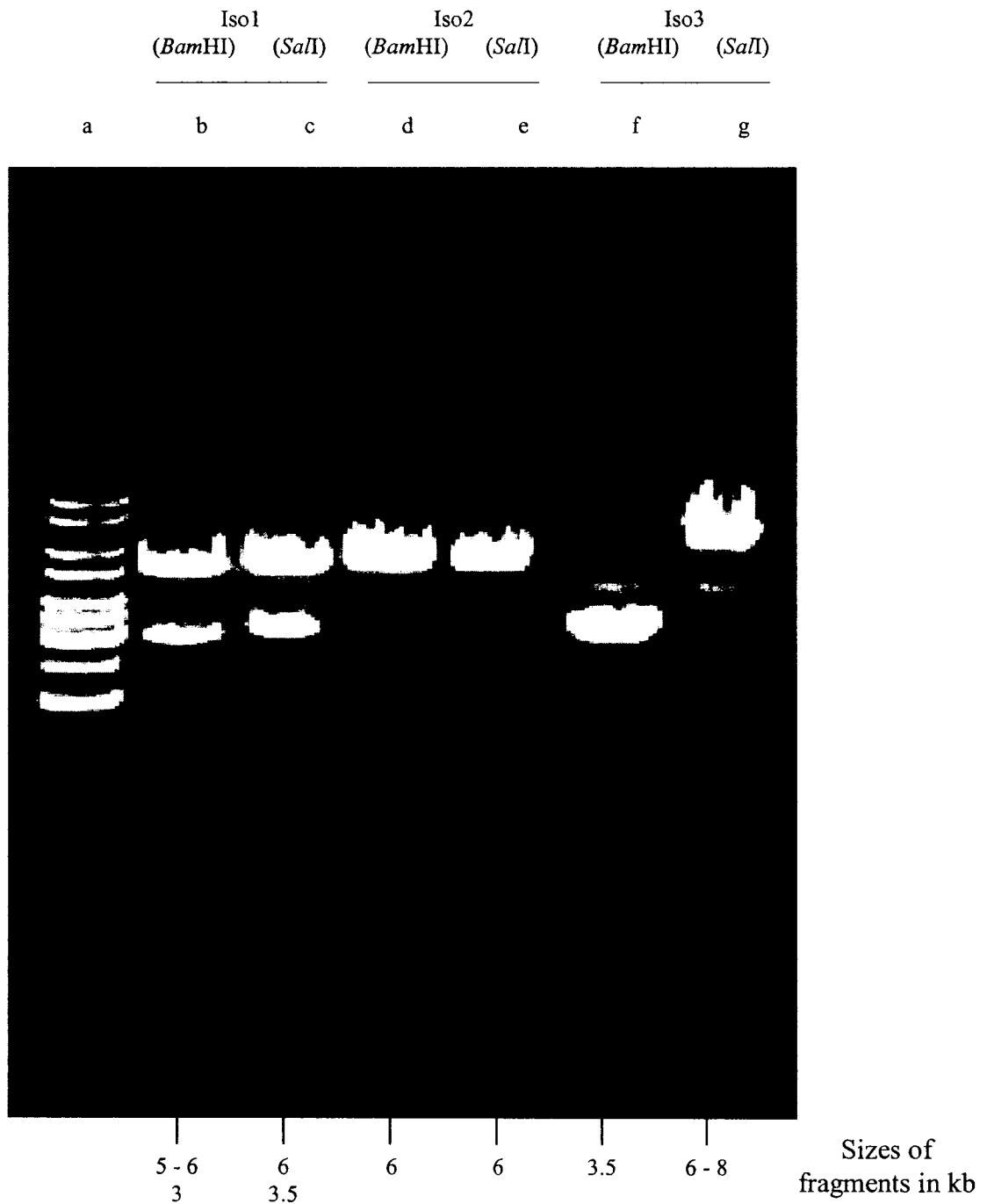


Fig. 4.5. Restriction analyses of *clpB* cloned into pGEM-T Easy Vector. Cloned isolates were screened from white colony growth and identification of products were done by cleaving suspected isolate plasmid constructs with *Bam*HI (b, d, f) and *Sal*I (c, e, g). Sequencing of PCR products confirmed presence of the *clpB* gene in pGEM-T Easy Vector plasmid of isolates 1 and 3. A 1kb DNA ladder, denoted by (a), was used as a marker.

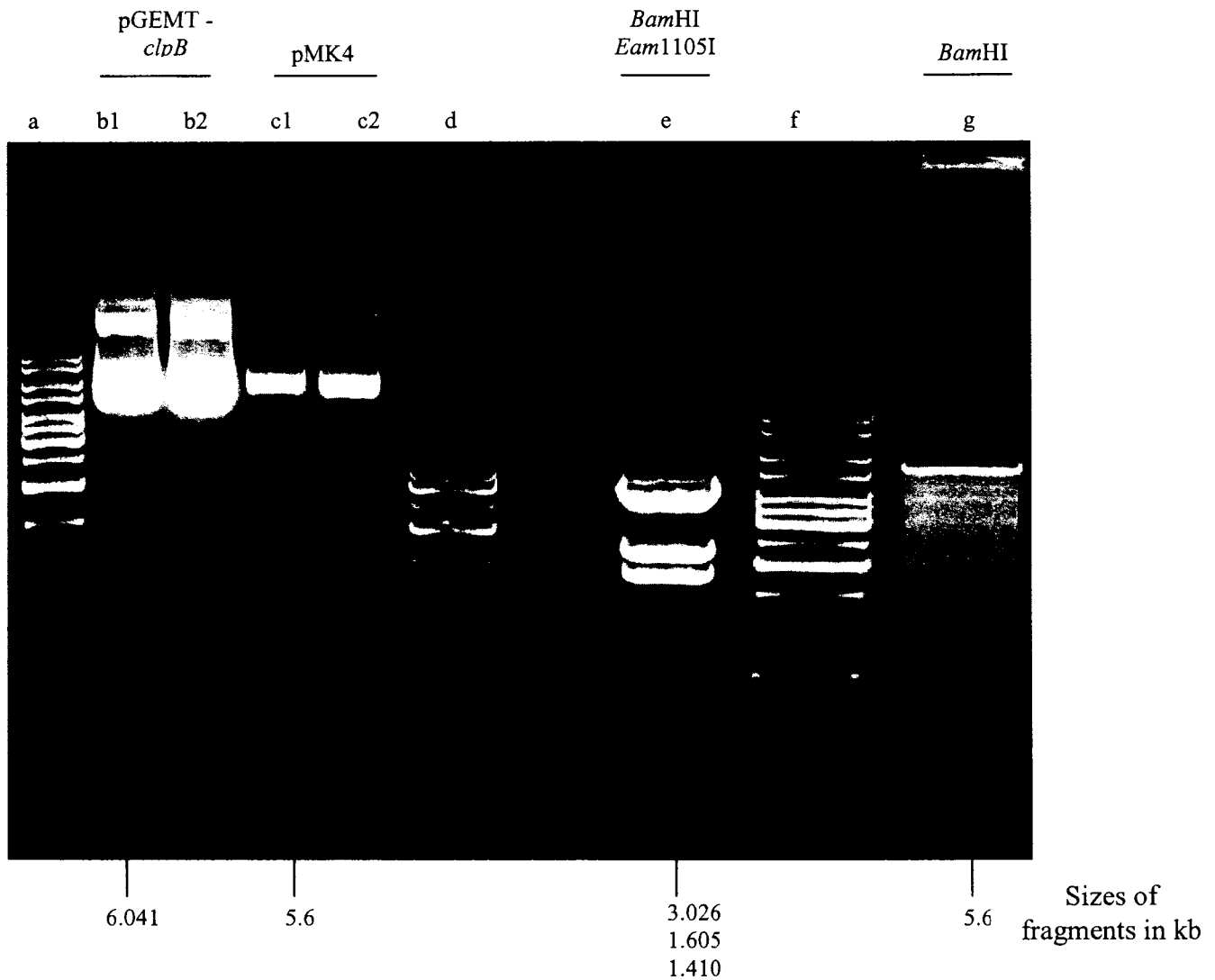


Fig. 4.6. Gel electrophoresis of plasmid extracts with their approx. band sizes are denoted by (b1, b2) and (c1, c2). Restriction analysis of plasmids pGEM-T (with *clpB*) and pMK4 are denoted by (e) and (g) respectively. A double digestion was performed on the pGEM-T Easy Vector to obtain *clpB* because of similar vector and insert sizes. (a), (d), and (f) are DNA ladders (1kb).



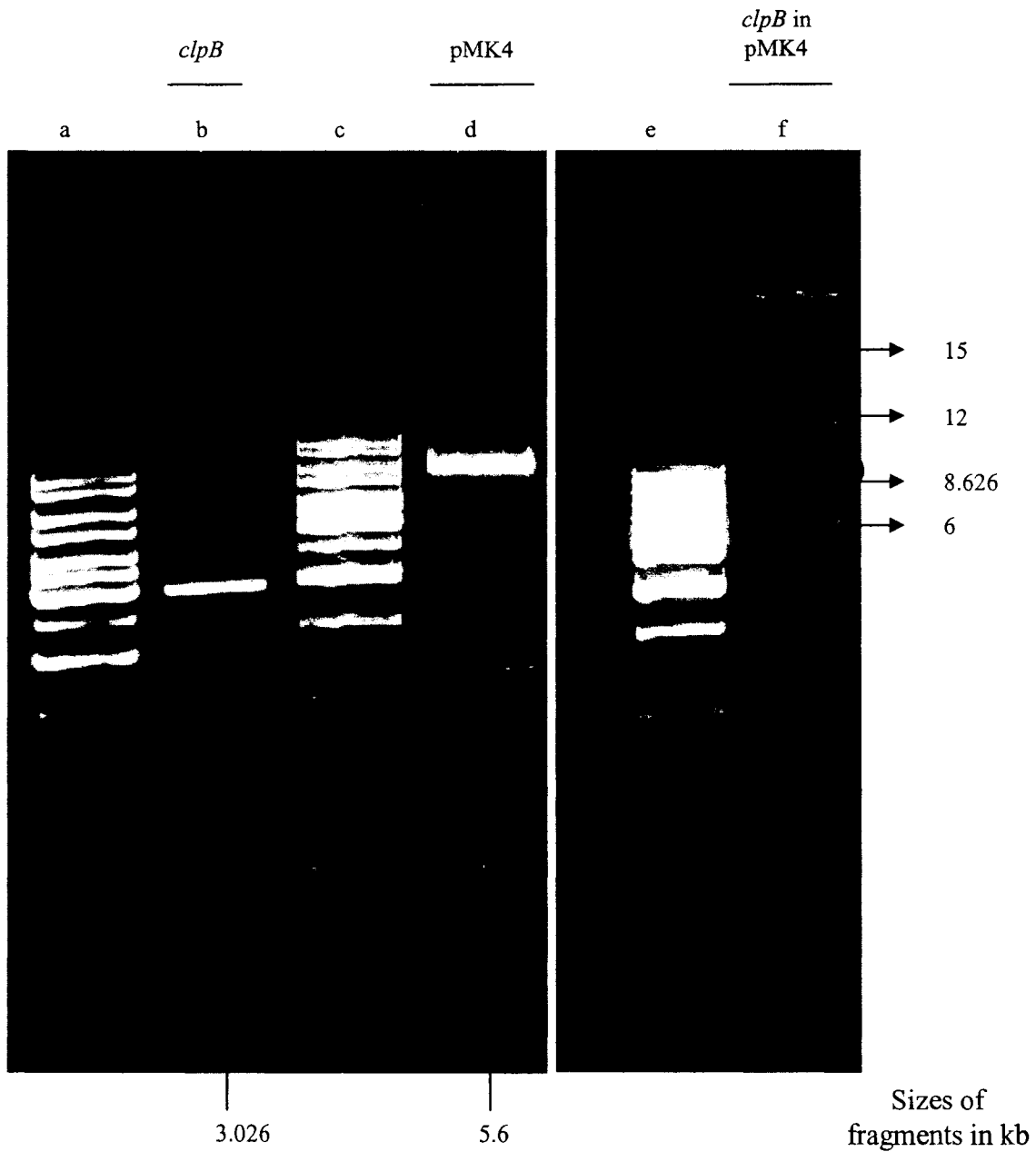


Fig. 4.7. Gel purified insert (b) and vector (d). Gel electrophoresis of ligated insert and vector denoted by (f). (a), (c), and (e) are DNA ladders (1kb).

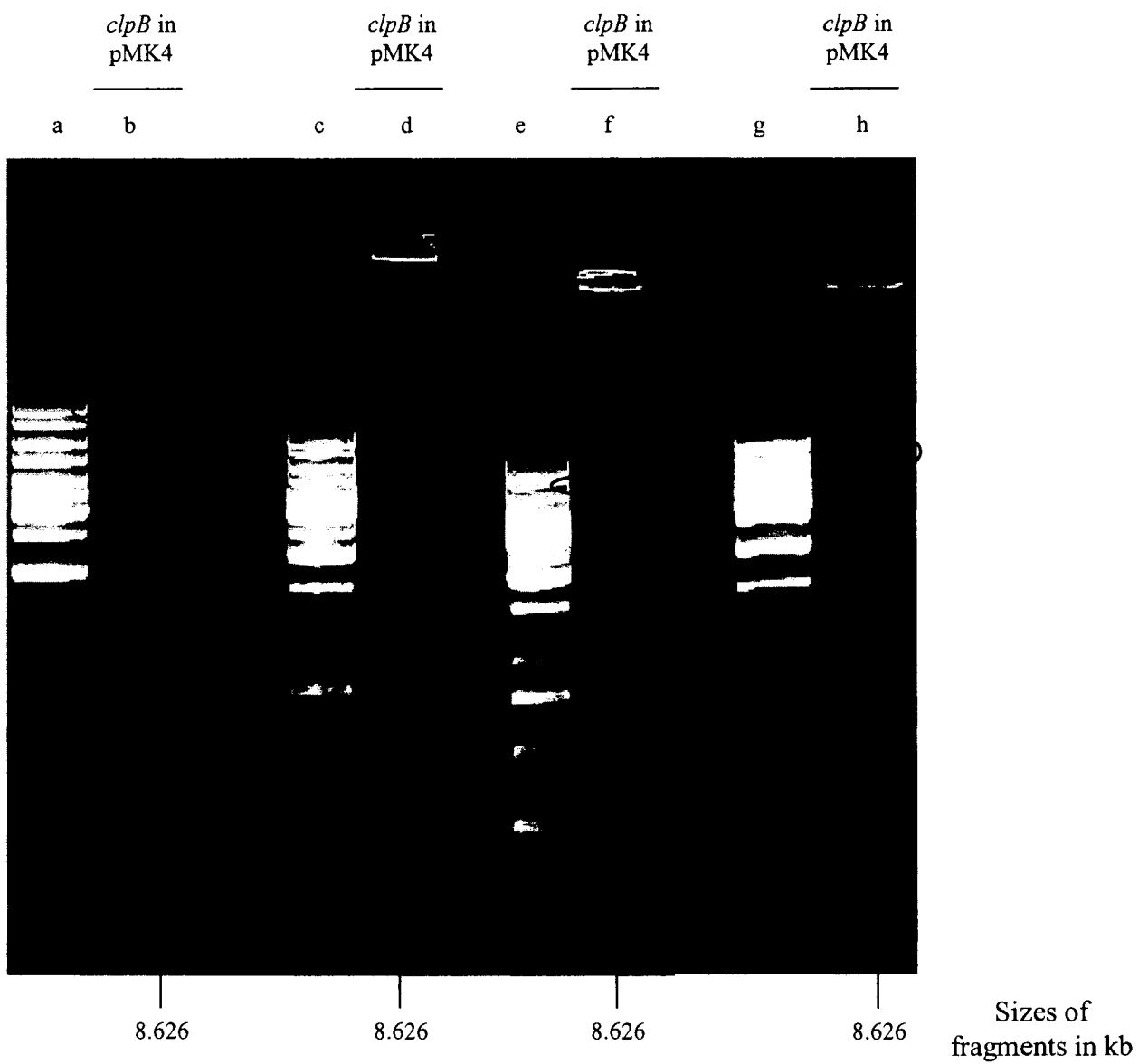


Fig. 4.8. Gel electrophoresis of ligated insert and vector denoted by (b), (d), (f), (h). (a), (c), (e), and (g) are DNA ladders (1kb).

## References

- Allan, E., P. Mullany, and Tabaqchali, S. 1998. Construction and characterization of a *Helicobacter pylori* clpB mutant and role of the gene in the stress response. *J. Bacteriol.* 180(2):426-429.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Chae, M. S. 2004. The role of flagella, physicochemical surface properties, and genes of *Listeria monocytogenes* in attachment and biofilm formation. PhD Thesis. Department of Food Science. University of Guelph.
- Chastanet, A., I. Derre, S. Nair, and Msadek, T. 2004. ClpB, a novel member of the *Listeria monocytogenes* CtsR regulon, is involved in virulence but not in general stress tolerance. *J. Bacteriol.* 186(4):1165-1174.
- Cossart, P., M. F. Vicente, J. Mengaud, F. Baquero, J. C. Perez-Diaz, and Berche, P. 1989. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. *Infect. Immun.* 57(11):3629-36.
- Dunn, B. E., R. M. Roop II, C. C. Sung, S. A. Sharma, G. I. Perez-Perez, and Blaser, M. J. 1992. Identification and purification of a cpn60 heat shock protein homolog from *Helicobacter pylori*. *Infect. Immun.* 60:1946-1951.
- Frees, D., A. Chastanet, S. Qazi, K. Sorensen, P. Hill, T. Msadek, and Ingmer, H. 2004. Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in *Staphylococcus aureus*. *Mol. Micro.* 54(5):1445-1462.
- Freitag, N. E. 2000. Genetic tools for use with *Listeria monocytogenes*, pp. 488-498. In Fischetti, V. A., R. P. Novick, J. J. Ferretti, D. A. Portnoy, and Rood, J. I. (ed.), Gram-positive pathogens. ASM Press, Washington, D.C.
- Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, Baquero, F., et al. 2001. Comparative genomics of *Listeria* species. *Science* 294:849-852.
- Gottesman, S. and Maurizi, M. R. 1992. Regulation by proteolysis: energy-dependent proteases and their targets. *Microbiol. Rev.* 56:592-621.
- Kaufmann, S. H. E. and Schoel, B. 1994. Heat shock proteins as antigens in immunity against infection and self, pp. 495-531. In Morimoto, R. I., A. Tissieres, and Georgopoulos, C. (ed.), The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

- Luchansky, J. B., P. M. Muriana, and Klaenhammer, T. R. 1988. Application of electroporation for transfer of plasmid DNA to *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Bacillus*, *Staphylococcus*, *Enterococcus* and *Propionibacterium*. *Mol. Microbiol.* 2:637–646.
- Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and Tauxe, R. V. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607-625.
- Nair, S., I. Derre, T. Msadek, O. Gaillot, and Berche, P. 2000. CtsR controls class III heat shock gene expression in the human pathogen *Listeria monocytogenes*. *Mol. Microbiol.* 35:800-811.
- Parsell, D. A., and Lindquist, S. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* 27:437-496.
- Porankiewicz, J. and Clarke, A. K. 1997. Induction of the heat shock protein ClpB affects cold acclimation in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J. Bacteriol.* 179:5111-5117.
- Squires, C. and Squires, C. L. 1992. The Clp proteins: proteolysis regulators or molecular chaperones? *J. Bacteriol.* 174:1081-1085.
- Suerbaum, S., J. M. Thiberge, I. Kansau, R. L. Ferrero, and Labigne, A. 1994. *Helicobacter pylori* hspA-hspB heat-shock gene cluster: nucleotide sequence, expression, putative function and immunogenicity. *Mol. Microbiol.* 14:959-974.
- Sullivan, M., R. Yasbin, and Young, F. 1984. New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. *Gene* 29:21–26.
- Vicente, M. F., F. Baquero, and Perez-Diaz, J. C. 1985. Cloning and expression of the *Listeria monocytogenes* haemolysin in *E. coli*. *FEMS Microbiol. Lett.* 30:77-79.
- Wirth, R., F. Y. An, and Clewell, D. B. 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli*-*S. faecalis* shuttle vector. *J. Bacteriol.* 165:831–836.

## 5. SUMMARY AND CONCLUSION

Bacterial cells for all five mutant strains of *L. monocytogenes* EGD were found attached to glass slides and all readily formed biofilms after 24 h. For all strains investigated, biofilms grown without shear force at 22°C had higher mean biofilm cell counts than at 37°C ( $P < 0.05$ ), although mutants M11 and M14 showed significantly reduced planktonic growth rates at 22°C over 24 h. At 37°C without shear force, only M11 was observed having significantly lower biofilm cell counts than its wild-type parent strain ( $P < 0.05$ ). When shear force was present, only mutant M7 produced significantly higher biofilm cell counts than its wild-type parent at 37°C ( $P < 0.05$ ). SEM imaging of biofilms grown at 37°C without shear force confirmed that the wild-type strain EGD produced significantly more biofilm cells than its mutant M11 (as observed with plate counts). Significantly more biofilm cells were also seen at 22°C than at 37°C for both strains tested. For biofilms grown at 22°C and dehydrated with hexamethyldisilazane (HMDS), both strains produced characteristic three-dimensional biofilms with cells embedded in what is believed to be extracellular polymeric substances (EPS). This presumed EPS matrix was not visible when 22°C biofilms were dehydrated with the critical point drying (CPD) method. The matrix was also not seen in biofilms grown at 37°C irrespective of the dehydrating method applied.

The PHLIP analysis of data obtained from CSLM showed mutants M7, M11 and M14 having greatly reduced biofilm biovolume and roughness compared to all other strains investigated at 22°C with shear force applied. At 37°C, with and without shear force, two strains (M11 and M14) showed the same trend. Results from PHLIP and one-way ANOVA corresponded with results from the principal components analysis (PCA). PCA found three strains (M7, M11, and M14) to be significantly different/ unique in structure from wild-type EGD. Mutant M7 stood out from other strains by producing biofilms high in biovolume, thickness and roughness at 22°C without shear force. M11 and M14 produced thicker and higher surface area to biovolume biofilms, at 22°C with shear and 37°C with and without shear. It was also found that the application of shear force reduced mean biofilm biovolume and roughness for all strains investigated.

A complementation *in trans* performed on mutant M11 (*clpB* absent) produced no positive clones after attempting a transformation into *E. coli* JM109. For this study, DNA concentration has been shown to be an important aspect concerning probability of attaining positive clones. Unfavorable low DNA concentration was attributed to using a low copy number plasmid, the large number of steps involved in our complementation procedure, and the spontaneous binding/joining of products (insert and vector) resulting in undesirable constructs during ligation.

In conclusion, investigating biofilms of *L. monocytogenes* mutants have shown that in the absence of certain genes, biofilm growth and structure formation can be affected significantly. Therefore, further research into gene manipulation should be done in order to fully understand the function of genes involved in biofilm formation.

There is also a need for a better understanding of *L. monocytogenes* biofilm growth and formation on common food contact surfaces. Such studies may not only aid in identifying prevention strategies, for example, modifications to food contact surfaces or sanitation practices, but also minimize *L. monocytogenes* biofilm production and formation in food processing environments.