MODULATION OF *Listeria monocytogenes* BIOFILMS BY *Pseudomonas* spp. SECRETED SUBSTANCES

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AHL	Acyl-homoserine lactone
EPS	Extracellular polymeric substances
HAA	3-(3-hydroxyalkanoyloxy)alkanoic acid
P. putida PCM	*Pseudomonas putida*- *Pseudomonas* conditioned medium
PAO1 PCM	*Pseudomonas aeruginosa* PAO1- *Pseudomonas* conditioned medium
PBS	Phosphate buffered saline
PCM	*Pseudomonas* conditioned medium
QS	Quorum sensing
Tet	Tetracycline
Tn	Transposon
Tnp	Transposase
TSA	Trypticase soy agar
TSB	Trypticase soy broth

Abstract

Listeria monocytogenes, a food-borne pathogen associated with severe disease, forms resistant biofilms which enable it to persist in food processing environments. Recently, surface active compounds and other microbially secreted substances have been reported to antagonize biofilm formation (Irie et al. 2005. FEMS Microbiol Lett 250, 237-243; Valle et al. 2006. Proc. Natl. Acad. Sci. U.S.A. 103, 12558-12563). These findings led us to investigate the use of secreted substances as a method for *Listeria* biofilm reduction. We screened isolates obtained from paper mill slimes for novel surfactant producing organisms, but were unable to find an isolate of interest. We then turned our investigation to the interactions between pseudomonads and *Listeria*. Conflicting reports regarding the influence of pseudomonads on the growth of *Listeria* biofilms led us to investigate the effect of substances secreted by *Pseudomonas putida* and *Pseudomonas aeruginosa* PAO1 on *L. monocytogenes* EGDe biofilms.

Listeria biofilms were grown on stainless steel and polystyrene at 22, 30, and 37°C and exposed to conditioned medium prepared from either *P. Putida* or *P. aeruginosa*. *P. putida* was found to secrete substances that significantly enhance *Listeria* attachment at 37°C. In contrast, *P. aeruginosa* was found to inhibit biofilm formation and disperse mature biofilms at temperatures ≤ 30°C. Treatment with *P. aeruginosa* conditioned medium reduced biofilms on stainless steel by 1.65 Log CFU/cm^2 and virtually eliminated all biofilm on polystyrene. The influence of temperature suggests that this reduction in biofilm formation may involve the flagella of *Listeria*.

To elucidate the genetic basis for this removal, 1416 random Tn5 mutants were screened for an altered ability to remove biofilm. One mutant was found to exhibit impaired biofilm removal, and it was determined that the transposon had disrupted the *lasB* gene, coding for the secreted metalloprotease elastase. These results indicate that elastase could potentially be developed as part of a novel antimicrobial strategy for the control of biofilms in the food industry.

1. Literature review

1.1 *Listeria monocytogenes* and listeriosis

Listeria monocytogenes is a ubiquitous environmental bacterium and a food borne pathogen that is responsible for one of the most serious food borne infections, listeriosis, which has a mortality rate of 30%. *L. monocytogenes* has been implicated in both sporadic and epidemic outbreaks, and although it accounts for just 1% of all cases of food-borne disease, it is responsible for 28% of food borne disease related deaths (Mead et al., 1999; Rocourt et al., 2001; Schuchat et al., 1991).

The majority of cases of listeriosis occur in individuals with impaired cell mediated immunity, such as pregnant women, the elderly and very young, and immunocomprimised individuals. In adults, invasive infection may result in meningitis, meningoencephalitis, and less commonly, abscesses of the brain or spinal cord or endocarditis (Farber & Peterkin, 1991). In pregnant women, infection is most common during the third trimester and can result in preterm labour, spontaneous abortions, or still birth. Neonatal disease from intrauterine infection can result in meningitis and has a mortality rate of 38% (Schuchat et al., 1991).

There are 13 serotypes of *L. monocytogenes*, yet only four of these are responsible for 98% of human listeriosis, and the majority of outbreaks and sporadic cases have been linked to serotype 4b (Buchrieser, 2007). *L. monocytogenes* has been described as having a Jekyll and Hyde personality, being well adapted to life as a saprophyte in soil and vegetation, yet in response to environmental cues is able to transform itself into a potentially deadly pathogen (Gray et al. 2006). Through complex regulatory pathways *L. monocytogenes* becomes a facultative intracellular pathogen

that can be engulfed by phagocytes, but it is also able to provoke engulfing by cells that are normally considered non-phagocytic. *L. monocytogenes* expresses two surface proteins, internalin A and B, which mediate its entry into host cells (Ireton, 2007). Once inside a cell, it leaves the phagocytic vacuole to replicate in the cytoplasm and uses actin based polymerization for motility and cell to cell spread (Ireton, 2007). Infection begins when *Listeria* enters intestinal epithelial cells, from which it can spread to the blood and into the liver, the major site of replication. Blood borne bacteria may also spread and infect the central nervous system (Gandhi & Chikindas, 2007; Gray et al. 2006).

Once *L. monocytogenes* is ingested by a host, it has several mechanisms to evade host defenses. During infection, flagella will provoke a strong immune response; therefore, suppression of flagella is believed to have evolved as a means of evading the host immune system. When *Listeria* encounters physiological temperature (37°C), it rapidly down regulates its expression of flagella. At 37°C, the motility gene repressor (MogR) blocks transcription of *flaA*, the flagellin subunit. MogR also represses transcription of its own anti-repressor, GmaR. However, at low temperatures a fourth protein, DegU, activates transcription of *gmaR* through an unknown mechanism, allowing GmaR to remove MogR, thus enabling production of flagellin (FlaA) (Shen & Higgins, 2006; Shen et al., 2006). *Listeria* also demonstrates a high level of acid tolerance, which enables it to survive the low pH of the stomach. Acid tolerance has been linked to virulence, and pre-adaptation to low pH values, such as those encountered in the stomach, has been reported to increase invasiveness and survival of *Listeria* (Gray et al. 2006).

Despite the serious illness that can result from infection with *L. monocytogenes,* the organism was not identified as a food-borne pathogen until 1981, following a large outbreak in Nova Scotia linked to consumption of coleslaw produced from contaminated cabbage (Schuchat et al., 1991). Since then, several large outbreaks have occurred linked to foods such as contaminated milk and soft cheeses. *L. monocytogenes* has been identified in nearly all types of foods; however, some of the more common ones include vegetables, soft cheeses, seafood, ice cream, pork, ready-to-eat meats and foods stored at refrigeration temperature for long periods of time (Rocourt et al., 2001; Schuchat et al., 1991; Thevenot et al., 2006; Zhu et al., 2005).

L. monocytogenes is a small, non-spore forming Gram positive rod. It is a facultative anaerobe and lives in variety of habitats, including soil, water, sewage, and decaying vegetation, and has been reported to survive in plant materials for up to 10-12 years (Gray et al. 2006; Schuchat et al., 1991). As it is widespread in the environment, *Listeria* can easily enter food processing settings and once there, several characteristics of its physiology enable it to persist. Foremost is its ability to survive and grow at refrigeration temperature. Although it has an optimum growth temperature of 30-37°C, it is capable of survival and growth at 1-45°C. Thus, refrigerated ready to eat foods are of particular concern (Gandhi & Chikindas, 2007). *Listeria* is also tolerant to osmotic stress and a wide range of pH values. However, the major factor in enabling its survival and persistence food processing environments is its ability to form resistant biofilms.

1.2 *Listeria* biofilms and biofilms in the food industry

Biofilms are communities of surface attached cells embedded within a matrix of extracellular polymeric substances (EPS). Compared to planktonic cells, those that are

part of a biofilm demonstrate increased resistance to desiccation, sanitizers and anti microbial agents. Biofilms within food processing environments are a major concern because they are difficult to prevent and remove, and they create a persistent risk of contamination.

Just as *Listeria* has a number of mechanisms that enable it to effectively invade and establish itself as a pathogen in host organisms, it is also well adapted for survival in the environment. While expression of flagella may be detrimental during infection, motility of the flagella has been reported to be critical to *Listeria* biofilm formation (Lemon et al., 2007), and at temperatures of 30°C or below *L. monocytogenes* becomes motile with peritrichous flagella. The expression of flagella enables *Listeria* to adhere to surfaces and initiate biofilm formation, and it has been demonstrated to form biofilms on a variety of materials commonly used in the food industry (Beresford et al., 2001). Some of the more problematic areas for biofilm formation include floors, bends in pipes, stainless steel surfaces, and conveyor belts (Kumar & Anand, 1998).

Once established, the *Listeria* biofilms can survive for long periods of time, becoming a persistent source of contamination. Detaching cells can contaminate foods before and after processing, and cross contamination may occur at any stage between the processing plant and the consumer, and contamination in the home is also a risk (Thevenot et al., 2006).

Biofilms and contamination are a continual problem in the food processing setting, and a number of strategies are currently used to control biofilm formation. Mechanical methods of biofilm removal include ultrasound, electrical fields, and combinations of these treatments with biocides or antibiotics (Kumar & Anand, 1998).

Other strategies include the use of detergents, anti-fouling paints containing silver, and food packaging materials containing edible films of antimicrobials (Gandhi & Chikindas, 2007; Kumar & Anand, 1998). Another approach is active packaging, in which materials are incorporated into the packaging to control the atmosphere and inhibit growth of pathogens on the food product (Gandhi & Chikindas, 2007). Substances of microbial origin, including bacteriocins, such as the antimicrobial peptide nisin, have been used both on food processing surfaces and directly in food products. Enzymes such as endoglycosidase and EPS degrading enzymes from *Streptomyces* have also been used in biofilm control (Kumar & Anand, 1998). Despite the variety of options available, biofilms in many industries remain problematic, and due to evidence of increasing rates of resistance to antibiotics, sanitizers, and bacteriocins, there is an ongoing effort to develop effective methods to prevent the growth of *Listeria* (Gandhi & Chikindas, 2007; [Anon], 2006).

1.3 Quorum sensing

There is evidence that many species require quorum-sensing for the construction of biofilms (Parsek & Greenberg, 2005). Quorum-sensing (QS) is a form of cell-cell communication that involves the release and detection of signalling molecules called autoinducers (Daniels et al., 2004; Waters & Bassler, 2005).

In Gram-negative species the most common autoinducers are acylated homoserine lactones (AHLs) (Whitehead et al., 2001). Although it is not a wide spread phenomenon, production of AHLs is a highly conserved regulatory system and approximately 4%, over 50 species, of Proteobacteria genera have been found to produce these molecules (Fuqua et al., 2001; Manefield & Turner, 2002).

The paradigm for AHL quorum-sensing is based on the LuxIR system found in *Vibrio fischeri*, the first of such systems to be described. The two main components of the system are the autoinducer synthase, LuxI, and the autoinducer receptor/DNA-binding transcriptional activator, LuxR. Newly synthesized autoinducers diffuse freely from the cell, and as the cell density increases, there is a corresponding increase in their extra-cellular concentration. The threshold concentration is reached once autoinducers begin binding to their receptor, LuxR. The LuxR-autoinducer complex then activates transcription of quorum-sensing controlled genes, including the one encoding LuxI. The result is a positive feedback loop that floods the extracellular space with autoinducers (Chen et al., 2005; Fuqua et al., 2001; Waters & Bassler, 2005).

The Gram positive counterpart to the AHL signalling systems of Gram-negative species is based on oligopeptide signals. Oligopeptides, which are 5-17 amino acids long, bind to membrane bound histidine kinase receptors and initiate a phosphorylation cascade that ultimately alters the activity of DNA-binding transcriptional regulatory proteins (Camilli & Bassler, 2006; Waters & Bassler, 2005).

Both the AHL and oligopeptide systems are believed to be designed predominantly for intraspecies communications due to the high level of specificity between the signals and their receptors (Waters & Bassler, 2005). In contrast, LuxS quorum sensing has been suggested to be an interspecies communication system. It is based on an autoinducer called AI-2 which has been identified in a wide variety of Gram-negative and Gram-positive species. AI-2 is produced by the LuxS autoinducer synthase, which produces 4,5-dihydroxy-2,3-pentanedione (DPD) that then undergoes a variety of spontaneous, species specific rearrangements (Camilli & Bassler, 2006). The

luxS gene is highly conserved among a diverse range of species; thus, it has been proposed to be a universal signal (McNab et al., 2003; Xavier & Bassler, 2003).

Natural mechanisms for QS signalling interference among prokaryotes, and between prokaryotes and eukaryotes, have been reported for a variety of species. Examples include the destruction of AHLs by *Bacillus* spp., inhibition of oligopeptide signalling between strains of *Staphylococcus aureus*, and destruction of AI-2 by *E. coli* (Camilli & Bassler, 2006). The evidence for interspecies communication has led to investigation of signal transduction interference as a novel method of biofilm control, and signal molecules such as AI-2 have been suggested as novel targets for biofilm prevention (Scheie & Petersen, 2004).

1.3.1 Quorum sensing in *Pseudomonas aeruginosa*

The complexities of quorum-sensing are illustrated by the systems operating in *Pseudomonas aeruginosa,* in which the expression of up to 400 genes is under QS control (Parsek & Greenberg, 2005). There are two distinct AHL quorum-sensing systems in *P. aeruginosa*, the LasIR system, the RhlIR system, and a third non-AHL system, which is based on the *Pseudomonas* quinolone signal (PQS) (Camilli & Bassler, 2006; Waters & Bassler, 2005).

The Las and Rhl AHL systems are arranged in a hierarchical series, and both are homologous to the LuxIR system of *V. fischeri* (Chen et al., 2005; Daniels et al., 2004; Latifi et al., 1996; Waters & Bassler, 2005).The LasIR system is the first to be activated, and is responsible for the activation of the RhlIR system (Latifi et al., 1996; Waters & Bassler, 2005). LasI synthesizes an AHL autoinducer, *N*-(3-oxododecanoyl)-$_L$-homoserine lactone, (3OC12)-HSL, which binds to LasR once it reaches the threshold

concentration. The LasR-(3OC12)-HSL complex then activates transcription of the genes controlled by the Las system, in addition to activating the RhlIR system. RhlI synthesizes a second AHL autoinducer, *N*-butanoyl-$_L$-homoserine lactone, (C4)-HSL, that binds to RhlR upon accumulating to the necessary threshold concentration (Chen et al., 2005; Daniels et al., 2004; Waters & Bassler, 2005; Whitehead et al., 2001). The RhlR-(C4)-HSL complex then activates transcription of its own set of target genes (Chen et al., 2005; Waters & Bassler, 2005; Whitehead et al., 2001). The expression of some genes is specific to either Las or Rhl QS, however, there are also many genes that are under the control of both systems (Nouwens et al., 2003).

An additional system, based on the *Pseudomonas* quinolone signal (PQS), is involved in the expression of virulence factors and biofilm formation, and is intertwined with both the Las and Rhl systems. PQS has been identified in *P. aeruginosa*, and PQS systems are also believed to present in other *Pseudomonas* spp. and *Burkholderia* spp. (Diggle et al., 2007).

1.4 Biosurfactants

While targeting QS itself, and by using signalling molecules, is one approach to biofilm removal or prevention, an alternative strategy is to investigate other secreted substances of microbial origin, many of which are regulated by QS.

Many of the known secreted substances produced by microorganisms that show potential for use in industry have surface active properties. Surface active agents are amphiphilic compounds characterized by the ability to form micelles and to lower surface and interfacial tension (Singh et al., 2007). Surfactants of microbial origin, referred to as biosurfactants, are synthesized by a wide variety of bacteria and yeast

genera and comprise an array of chemical structures including glycolipids, lipopeptides, lipoproteins, phosopholipids, fatty acids, and neutral lipids (Rodrigues et al., 2006a).

The majority of known biosurfactants are glycolipids, and examples include rhamnolipids produced by *Pseudomonas* spp., trehalolipids produced by species belonging to *Mycobacteria* and *Nocardia*, and sophorolipids produced by several species of yeast (Desai & Banat, 1997). Lipopeptide and lipoprotein biosurfactants have also been identified, many of which have antibiotic properties and high surface activities. Examples of these types of compounds include the surfactants surfactin and iturin, both produced by *Bacillus subtilis*, *Serratia* produced serrawettins, and gramicidin and polymyxin antibiotics produced by *Bacillus brevis* and *Bacillus polymyxa* respectively (Ahimou et al., 2000; Rodrigues et al., 2006a; Tanikawa et al., 2006).

The physiological roles of biosurfactants are not well understood and few have been studied in detail (Van Hamme et al., 2006). The diverse nature of the compounds, and the organisms producing them, make generalizations difficult and the various types of surfactants likely have different roles (Rodrigues et al., 2006e; Ron & Rosenberg, 2001). To date, surfactants have been found to be involved in a wide range of functions, including the uptake of water-insoluble substrates, motility and swarming, amensalism, virulence, biofilm formation and maturation, and in many cases numerous functions have been identified for a single compound (Rodrigues et al., 2006b; Ron & Rosenberg, 2001; Van Hamme et al., 2006).

1.4.1 Rhamnolipid biosynthesis

Some of the most studied substances secreted by *P. aeruginosa*, and the best characterized of all biosurfactants, are rhamnolipids (Desai & Banat, 1997; Maier &

Soberon-Chavez, 2000; Rodrigues et al., 2006a). Rhamnolipid production is controlled by the Rhl QS system (which is in turn controlled by the Las and PQS systems) and involves the addition of rhamnose to the hydrophobic fatty acid moiety, 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) (Chen et al., 2004; Ochsner & Reiser, 1995; Pearson & Hansen, 2007; Pearson et al., 1997). The genes involved in rhamnolipid synthesis are arranged in two operons, with *rhlA, rhlB, rhlR,* and *rhlI* forming one cluster, while the *rhlC* gene is located elsewhere on the chromosome in an operon with a second gene (PA1131) whose function is unknown (Soberon-Chavez et al., 2005).

There are three main proteins involved in rhamnolipid synthesis: RhlA, RhlB, and RhlC. RhlA is involved in the synthesis of HAAs (Deziel et al., 2003). These molecules are both an end product and an intermediate, as some HAAs are released from the cell without further modification, while others will have either one or two rhamnose moieties attached to become mono or dirhamnolipids prior to being excreted. RhlB is a membrane bound rhamnosyl transferase that produces monorhamnolipids through the addition of dTDP-$_L$-rhamnose to an HAA (Soberon-Chavez et al., 2005). Like their precursor HAA, monorhamnolipids may either be excreted from the cell or have a second rhamnose added to produce a dirhamnolipid. This reaction is carried out by RhlC, a second membrane bound rhamnosyl transerferase which adds dTDP-$_L$-rhamnose to monorhamnolipids (Rahim et al., 2001). The production of rhamnolipids is illustrative of the complex networks of QS hierarchies in *Pseudomonas*, and the number of interacting factors that may be involved in the synthesis of secreted substances.

1.4.2 Physiological role

Several physiological functions for these biosurfactants have been identified to date. A number of studies have suggested that the physiological role of biosurfactants, including rhamnolipids, involves the emulsification and uptake of water immiscible substrates. However, there is evidence indicating that this is not their primary function (Desai & Banat, 1997; Francy et al., 1991; Guerrasantos et al., 1986; Koch et al., 1991; Ochsner et al., 1994).

Conflicting with the assumption that biosurfactants are produced to enable growth on hydrocarbons, is the finding that rhamnolipids are produced most efficiently when grown with water soluble substrates, and a linear increase in production is observed with increased amounts of glucose (Guerrasantos et al., 1986). Similarly, when screening for surfactant producing organisms, glucose has been found to be the substrate that will support surfactant production in the greatest number of species (Batista et al., 2006). Another soluble substrate, glycerol, has been identified as the optimal substrate for rhamnolipids production, giving higher yields than other soluble carbon sources or *n*-alkanes (Arino et al., 1996). Furthermore, Beal et al. (2000) found that rhamnolipids provide only a minor enhancement in hexadecane uptake, that they are not necessary for the process to occur, as a rhamnolipid deficient mutant assimilated 69% of the hexadecane taken up by the wild type strain. The fact that rhamnolipids are produced during the stationary phase of growth further contradicts the idea that their role is in substrate uptake (Arino et al., 1996).

In an attempt to reconcile these findings with the presumed natural function of assimilation of insoluble substrates, it has been suggested that the high production of

rhamnolipids using glucose is an example of the over-production of seemingly unnecessary metabolites as a result of the cultivation conditions (Guerrasantos et al., 1986). However, as was pointed out by Van Hamme et al. (2006), it may be difficult to rationalize the difference between industrial applications of biosurfactants and their natural roles in microbial physiology. The most probable explanation appears to be that the primary function of these molecules is not related to substrate bioavailability (Arino et al., 1996; Soberon-Chavez et al., 2005). In contrast, there is evidence that rhamnolipids are involved in a range of functions including swarming and motility, maintenance of biofilm structure and dispersal, and they have antimicrobial properties that are effective against a range of other microorganisms (Caiazza et al., 2005; Pamp & Tolker-Nielsen, 2007).

1.5 Potential uses for biosurfactants

Studies involving biosurfactants indicate that they have potential for use in a broad range of industries. The most common examples are applications involving oil processing and microbial enhanced oil recovery (MEOR), or bioremediation of soil and water with hydrophobic or metal contaminants (Banat et al., 2000; Batista et al., 2006; Bodour et al., 2003; Mulligan, 2005; Plaza et al., 2006; Singh & Cameotra, 2004; Singh et al., 2007). Biosurfactants are well suited to bioremediation because they can be synthesized *in situ* and are less toxic than chemical surfactants, which due to their toxicity may represent an additional source of contamination (Batista et al., 2006).

Another major area of research is in biomedical sciences. A number of biosurfactants have been shown to have antiviral, antifungal, or antibacterial activities

(Singh & Cameotra, 2004). Surfactin, a biosurfactant produced by *Bacillus subtilis*, shows potential for use as a thrombolytic agent for both urgent disorders and long term use (Singh & Cameotra, 2004). *B. subtilius* has also been found to exhibit antitumour activity, and a variety of glycolipid surfactants have been shown to induce cell differentiation, growth arrest, and apoptosis instead of proliferation in mouse malignant cells (Cameotra & Makkar, 2004; Isoda et al., 1997; Rodrigues et al., 2006a; Singh & Cameotra, 2004). Lipopeptide surfactants have been reported to act as immunomodulators and to inhibit specific enzymes (Rodrigues et al., 2006a). A number of biosurfactants have been shown to prevent adhesion of pathogenic organisms, and have potential for use as a coating for catheters or prosthetic devices to prevent infection and biofilm formation (Rodrigues et al., 2006a; Rodrigues et al., 2006b; Rodrigues et al., 2006c; Rodrigues et al., 2006d; Rodrigues et al., 2006e; Rodrigues et al., 2004a; Rodrigues et al., 2004b). Additional areas where biosurfactants show potential for use include agriculture, cosmetics, food, and manufacturing industries (Banat et al., 2000; Singh et al., 2007).

1.6 Objectives of this study

The objective of our study is to identify substances of microbial origin that could potentially be used to control *Listeria monocytogenes* biofilms in food-processing settings, thereby reducing the risk of food-borne disease. We started our study by attempting to identify novel surfactant producing organisms, and screened bacterial isolates obtained from paper mill slimes in search of biosurfactant producers. As paper mill slimes have been found to consist of a variety of bacterial species that rapidly

produce large amounts of EPS and biofilm (Chaudhary et al., 1997), we hypothesized that the slimes could be a source of isolates that secrete biosurfactants, which could then be tested for the ability to prevent or reduce *Listeria* biofilms.

Next, we focused our work on two *Pseudomonas* species, *P. aeruginosa* PAO1 and *P. putida*, to determine if substances secreted by these organisms are capable of removing *Listeria* biofilms, as well as to gain a better understanding of how substances secreted by *Pseudomonas* spp. modulate the growth of *Listeria* biofilms under a range of growing conditions. As *Listeria* and *Pseudomonas* are both common environmental species, they encounter each other in the environment and studies investigating the effect of *Pseudomonas* species on the growth of *Listeria* biofilms have produced conflicting results (Carpentier & Chassaing, 2004; Guobjornsdottir et al., 2005; Norwood & Gilmour, 2001).

Our investigation revealed that *Pseudomonas aeruginosa* PAO1 conditioned medium inhibited *Listeria* biofilms, thus our final objective was to identify the compounds involved. By screening a library of *P. aeruginosa* random knockout mutants, we determined that the enzyme elastase (LasB) plays a role in preventing and removing *Listeria* biofilms.

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2. Screening pulp and paper isolates for a novel biosurfactant producing organism

2.1 Introduction

Potential applications for microbial surfactants have been identified in a wide range of industries and there has been increasing interest in their commercial development. Biosurfactants offer several advantages over chemical surfactants, including mild production conditions, lower toxicity, higher biodegradability, and effectiveness at extreme temperatures, pH values, and salinity (Plaza et al., 2006; Rodrigues et al., 2006a). They may also possess unique structures, not found among chemical surfactants (Maier & Soberon-Chavez, 2000). However, few biosurfactants have actually been used on an industrial scale due to the lack of economical production processes, and there is an ongoing search for cost effective surfactant producers (Batista et al., 2006).

Despite their recognized potential, the distribution of surfactant producing organisms in the environment is not well understood and there have been few studies aimed at screening for biosurfactants and the organisms that make them (Bodour & Miller-Maier, 1998; Bodour et al., 2003). Bodour et al. (2003) conducted one of the few screening studies that have been published to date, which examines the distribution of biosurfactant-producing bacteria in undisturbed and oil/metal contaminated soils in the South-western United States. Other studies screening for biosurfactant producers typically involve environments where the surfactant producing organisms could be used. These include locations such as hydrocarbon contaminated soil and water (Batista et

al., 2006; Bonilla et al., 2005; Chang et al., 2005) petroleum sludge (Dubey & Juwarkar, 2001; Plaza et al., 2006), and dairy equipment (Busscher et al.,1996).

As there are no common genes among surfactant producers, molecular approaches are not applicable, and screening must be done using other techniques such as surface tension analysis (Bodour et al., 2003). The most widely used method for the measurement of surface tension is the du Nouy ring method, which measures the force needed to pull a platinum wire ring through an interface. The criteria used to determine if an organism produces biosurfactant is the ability to reduce the surface tension below 40 mN m^{-1} (Batista et al., 2006). Although accurate, the technique is not well suited to screening large numbers of isolates because it is time-consuming, requires specialized equipment, and uses a relatively large volume of sample (Bodour & Miller-Maier, 1998; Busscher et al., 1996; Youssef et al., 2004). Other methods used to test for the presence of surfactants that are more amenable to screening studies include the drop-collapse method, blood agar lysis, oil spreading, and axisymmetric drop shape analysis (Busscher et al., 1996; Youssef et al., 2004).

In this study, the drop-collapse method was used to test for surfactant production by bacterial isolates obtained from a paper mill. The pulp and paper industry provides an ideal environment for bacterial growth due to the constant supply of water and nutrients, and the varied conditions, such as a range in temperature and pH values and the use of various additives, should result in the development of diverse microbial communities (Chaudhary et al., 1997). The aim of our study was to screen paper mill biofilms for novel surfactant producing organisms.

2.2 Methods

Cultures of 80 bacterial isolates obtained from paper mill slimes were tested for their ability to produce biosurfactants using the qualitative drop-collapse technique (Bodour & Miller-Maier, 1998; Jain et al., 1991). Each isolate was tested after growth in 10 ml of R2A broth (Becton Dickinson Difco, Sparks MD) and in 10 ml of R2A broth supplemented with 2% glucose (Batista et al., 2006; Bodour et al., 2003). The cultures were grown in glass test tubes incubated for 24h at 30°C. Tests were performed in the polystyrene lid of a 96-well microtiter plate (Costar 3596). To each well, 1.8 µl of 10W-40 oil (Penzoil, Oil City, PA) was added and allowed to equilibrate for 24 h at room temperature (Batista et al., 2006; Bodour et al., 2003). A sterile pipette was used to dispense 5 µl aliquots of the cell suspensions into the centre of the wells and each isolate was tested in triplicate. Drops that remained beaded were considered negative for the production of biosurfactants, while drops that spread out and collapse were considered positive. A 10^{-2} dilution of Tween 20 (Fisher BioReagents) was used as a positive control and ddH_2O was used as a negative control. To maximize the likelihood of finding a surfactant producing organism and to increase efficiency, the tests were conducted using cell suspensions, rather than filtered supernatants. Biosurfactants can be either adhered to, or an integral part of, a cell surface, thus cell suspensions are used for initial screening, and the cell free supernatant of those that produce a positive result can be tested to reveal whether the surfactant is released into the medium (Batista et al., 2006; Bodour & Miller-Maier, 1998; Bodour et al., 2003; Youssef et al., 2004).

2.3 Results and Discussion

All 80 isolates screened for the ability to reduce surface tension produced a negative result. It is possible that this result represents the true nature of the biofilms, and that the paper mill slimes tested here are not from an environment that supports surfactant producing organisms. However, there are many variables affecting surfactant production, and the method used to detect surfactant production, the growth medium, and the incubation length and temperature would all impact surfactant production and its detection.

The drop-collapse test was used to test for the presence of biosurfactants. This technique is based on the principle that a drop of a polar liquid applied to a hydrophobic surface will form a bead, while a liquid containing a surfactant will collapse and spread due to the reduced interfacial tension (Bodour & Miller-Maier, 1998; Jain et al., 1991). Youssef et al. (2004) compared methods of testing for biosurfactants and found that the drop-collapse technique gave reliable results, with false negatives limited to cultures containing only low levels of surfactant. Although it was found to be less sensitive than the oil-spreading technique, the drop-collapse method is recommended as an effective first line of screening to identify cultures that produce moderate to high levels of surfactant (Youssef et al., 2004). Thus, it is possible that some of the isolates were producing low levels of a surfactant that went undetected due to the limited sensitivity of the test. The oil-spreading technique may have been able detect dilute surfactants that were missed by the drop-collapse technique; however, the utility of such organisms as a source of biosurfactants is questionable (Youssef et al., 2004).

Surfactant production is dependent on the type of carbon source used as well as the types and amounts of other nutrients present in the growth medium (Bodour et al., 2003; Davis et al., 1999; Mukherjee & Das, 2005; Nitschke et al., 2005). In this study, glucose was chosen as the carbon source as it has been found to support the production of a variety of surfactants (Batista et al., 2006; Bodour et al., 2003). Had a different carbon source been used, it is possible that a surfactant producing organism may have been detected.

Another factor that may have impacted the results was the length of incubation. There is considerable variation among species in the phase of growth in which the onset of surfactant production occurs. Strains of *Lactobacillus* have been found to synthesize surfactants mainly during the first four hours of growth (Rodrigues et al., 2006c), while in other species, such as *P. aeruginosa*, biosurfactant production is not initiated until the stationary phase (Batista et al., 2006; Ron & Rosenberg, 2001). It is possible that the 24 h incubation period used in this study was insufficient for biosurfactant production to occur in some of the isolates, and the results may have been different had the isolates been incubated for an extended period of time. Although some studies use incubation times of up to seven days (Bodour & Miller-Maier, 1998; Plaza et al., 2006), Batista et al. (2006) speculated that prolonged incubation times may lead to degradation of the surfactants and suggested testing for surfactant production after 24-48 h.

Another explanation for why a biosurfactant producing organism was not detected may be that the sample size was too small. Results from studies that screen for such organisms suggest that these species may comprise a relatively small portion

of the culturable bacteria. Batista et al. (2006) screened 192 isolates from contaminated soil and water samples, and found that 19 (9.9%) of the isolates tested positive for surfactant production using the drop-collapse technique, and of these only five isolates secreted their surfactants into the culture medium. Boudour et al. (2003) screened 1305 isolates obtained from contaminated and undisturbed soils and found 45 putative surfactant producers, or 3.4% of the isolates.

Rather than continuing to screen pulp and paper isolates for novel surfactant producers, a decision was made to choose an organism that had been previously identified to produce biosurfactants for the remainder of the study. The strain that was selected was *Pseudomonas aeruginosa* PAO1, which produces rhamnolipids, a glycolipid biosurfactant. *P. aeruginosa* rhamnolipids were chosen because they are the best characterized of all biosurfactants, and their chemical structure and properties, as well as their biosynthesis, have been studied in detail (Desai & Banat, 1997; Maier & Soberon-Chavez, 2000; Rodrigues et al., 2006a). In addition to this, Irie *et al.* (2005) found that rhamnolipids were capable of dispersing *Bordetella bronchiseptica* biofilms after 4 hours of exposure to *P. aeruginosa* PAO1 conditioned medium. Similarly, Rodrigues et al. (2006b) found that an adsorbed rhamnolipid surfactant layer will prevent the adhesion, and therefore biofilm formation, of bacteria and yeasts on silicone rubber.

The PAO1 strain of *P. aeruginosa* was chosen because it has been used in a number of studies involving rhamnolipids (Boles et al., 2005; Davey et al., 2003; Irie et al., 2005) and its genome has been sequenced (Stover et al., 2000).

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3. Substances secreted by *Pseudomonas putida* and *Pseudomonas aeruginosa* PAO1 modulate the growth of *Listeria monocytogenes* biofilms

3.1 Introduction

Listeria monocytogenes, the etiologic agent of listeriosis, is a pathogenic bacterium associated with food borne disease outbreaks characterized by widespread distribution, hospitalization rates of over 90%, and mortality rates of 20 – 40% (Borucki et al., 2003; Farber & Peterkin, 1991; McLauchlin et al., 2004; Mead et al., 1999). The principle route of infection is ingestion of contaminated foods, and those most susceptible to the disease include the elderly, pregnant, and immunocompromised individuals (Gandhi & Chikindas, 2007).

L. monocytogenes has been found to contaminate a wide range of foods, including a variety of dairy products, meats, eggs, seafood, and vegetables (Farber & Peterkin, 1991). Its control in food processing environments is difficult due to its ability to grow at refrigeration temperatures (2-4°C), adapt to high salt concentrations, and to survive in acidic foods (Gandhi & Chikindas, 2007). Certain strains have been found to persist in food processing environments for extended periods of time and this persistence is aided by *Listeria's* ability to form biofilms (Holah et al., 2002; Pan et al., 2006). Biofilms are multispecies communities of microorganisms attached to a surface, and are particularly problematic due to their increased resistance to desiccation, disinfectants, sanitizing agents, and UV light compared to their planktonic counterparts (Costerton et al., 1999; Gandhi & Chikindas, 2007; Meylheuc et al., 2006). Within food processing environments biofilms can serve as a continual reservoir of bacteria,

creating problems of cross-contamination and post-processing contamination (Gandhi & Chikindas, 2007; Kumar & Anand, 1998; Pan et al., 2006).

It is hypothesized that the resident micro-organisms of food processing environments have a major impact on the likelihood of finding *Listeria*, and that it may be properties of the extracellular polymeric substances (EPS) secreted by these organisms that are responsible (Bremer et al., 2001; Carpentier & Chassaing, 2004; Leriche & Carpentier, 2000). *L. monocytogenes* biofilms have been reported to be either enhanced (Bremer et al., 2001; Carpentier & Chassaing, 2004) or prevented (Carpentier & Chassaing, 2004; Leriche & Carpentier, 2000; Norwood & Gilmour, 2001; Zhao et al., 2004) by the presence of other organisms; and even studies limited to investigating the effect of *Pseudomonas* species have produced conflicting results (Carpentier & Chassaing, 2004; Guobjornsdottir et al., 2005; Norwood & Gilmour, 2001).

Knowledge of how resident organisms modulate *Listeria* biofilm formation is an important aspect of their control. Recognition of species that are capable of enhancing the growth of *Listeria* may help to predict conditions that favour biofilm formation. On the other hand, species that inhibit the growth of *Listeria* may help to provide insight into strategies to control and prevent its growth. For example, Zhao et al. (2004) suggested the use of competitive-exclusion organisms to control *L. monocytogenes* biofilms, while a number of studies have investigated the use of non-antibiotic microbially secreted compounds, often with surface active properties, to prevent or eliminate biofilm formation by a variety of Gram negative and Gram positive species. It has also been reported that the adhesion of a variety of bacteria and yeasts could be prevented by preconditioning silicone rubber with rhamnolipid biosurfactant produced by

Pseudomonas aeruginosa (Rodrigues et al., 2006b), and also with surfactants produced by *Lactococcus lactis* (Rodrigues et al., 2004) or *Streptococcus themophilus* (Rodrigues et al., 2006a). In addition to preventing biofilm adhesion and formation, there is also evidence to indicate that such compounds are able to disrupt mature biofilms. For example, Irie et al. (2005) found that *Pseudomonas aeruginosa* PAO1 spent medium was able to disrupt *Bordetella bronchiseptica* biofilms after 10 minutes of exposure, and eliminated large portions of biofilm after 4 h. Likewise, Mireles et al. (2001) demonstrated that a surfactant produced by *Bacillus subtilis*, surfactin, is able to both prevent and disrupt biofilms of *Salmonella enterica*.

In this study, the effect of substances secreted by *Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* on *Listeria monocytogenes* EGD biofilms was investigated. Pseudomonads are commonly found in food processing environments and their ability to modulate *Listeria* biofilm formation is a concern from a food safety standpoint. As it has been shown that *Listeria* exhibits differential, temperature dependent adherence and biofilm formation, the impact of the *Pseudomonas* spp. spent medium on the growth of *Listeria* biofilms was examined at 22, 30, and 37°C on two common food industry materials, stainless steel and polystyrene (Chavant, et al., 2002; Ells & Hansen, 2006; Lemon et al., 2007; Norwood & Gilmour, 2001).

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

Strains used in this study included: *Listeria monocytogenes* EGD, *Pseudomonas aeruginosa* PAO1, and *Pseudomonas putida* LV-4, an isolate from a biofilm in a milk processing line (Chumkhunthod et al., 1998). Cultures were grown in either trypticase

soy broth (TSB; Becton Dickinson BBL, Sparks MD) or on trypticase soy agar (TSA; Becton Dickinson BBL, Sparks MD). Isolates were stored in TSB with 25% glycerol (v/v) at -80°C, and working cultures were maintained on TSA at 4°C.

3.2.2 Production of *Pseudomonas* conditioned medium (PCM)

Experiments were carried out with two *Pseudomonas* species: *P. aeruginosa* PAO1 and *P. putida*, which were used to prepare *P. aeruginosa* PAO1 conditioned medium (PAO1 PCM) and *P. putida* conditioned medium (*P. putida* PCM).

PAO1 PCM was prepared by growing *P. aeruginosa* PAO1 cultures in 80 ml of TSB for 48 hours at 37°C. Cells were removed by centrifugation at 14,000 x g at 4°C for 30 minutes, and the supernatant was filtered through a 0.22 µm filter. The filtered supernatant was then mixed with an equal volume of 2x concentrated TSB (Irie et al., 2005; Valle et al., 2006).

P. putida PCM was prepared as described above, from cultures of *P. putida* grown in 80 ml of TSB for 48 hours at 30°C.

3.2.3 Formation of biofilms on stainless steel

3.2.3.1 Preparation of stainless steel

Biofilms were grown on 1 cm^2 stainless steel coupons (#316). Prior to use, the coupons were prepared as described by Meylheuc et al. (2001). Briefly, the coupons were soaked for 15 minutes in a 2% (v/v) solution of RBS 35 Concentrate detergent (Pierce, Rockford IL), rinsed three times for five minutes with water at 50°C, and then rinsed three times with distilled demineralised water at 20°C. The coupons were then placed in glass Petri dishes and sterilized by autoclaving at 120°C for 20 minutes.

3.2.3.2 Biofilm growth and treatment with PCM

The effect of substances secreted by *Pseudomonas* spp. on *Listeria* biofilms was examined by introducing either PAO1 PCM or *P. putida* PCM to *Listeria* biofilms at different times after biofilm initiation (0, 3, 24 h), as outlined in Table 1. This approach enabled investigation of the effect of PCM on the biofilms at three stages of development: (1) initial cell attachment (2) growing biofilms and (3) mature biofilms.

L. monocytogenes biofilms were grown on the stainless steel coupons as described by Chae and Schraft (2000), with the following modifications. *Listeria* cultures were grown in TSB at 37°C for 24h, washed twice in phosphate buffer saline (PBS; 137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) by centrifugation at 3,000 x g at 4°C for 10 min, and standardized to OD_{600} = 0.32 ± 0.01 (corresponding to 10^8 cfu/ml) using a spectrophotometer. A sterile pipette was used to dispense 20 ml of the standardized cell suspension into sterile glass Petri dishes each containing three stainless steel coupons. The Petri dishes were then placed into larger Petri dishes containing moist paper towels to minimize evaporation, and incubated at either 37°C or 22°C with shaking at 90 rpm for 3 h to allow adhesion to occur. Following the 3 h incubation, non-adherent cells were removed by gently washing the steel coupons 2 times with 25 ml of PBS using a sterile pipette, and the biofilms were then supplemented with 20 ml of fresh medium.

To study the impact of PAO1 PCM and *P. putida* PCM on initial attachment and *Listeria* biofilm formation, biofilms were grown using either PAO1 PCM or *P. putida* PCM throughout the entire incubation period (Table 1A). The standardized cell suspensions were prepared in PCM initially, and fresh PCM was added following the 3 h

adhesion period. Controls were prepared using TSB in place of PCM. The biofilms were incubated for a total of 24 h.

To examine the impact on a growing biofilm, the PCM was introduced 3 h after biofilm initiation. *Listeria* biofilms were prepared in TSB and incubated for 3 h, at which time they were washed twice with 25 ml of PBS using a sterile pipette. The biofilms were then supplemented with 20 ml of either PAO1 PCM or *P. putida* PCM and incubated for an additional 21 hours, control biofilms were supplemented with fresh TSB (Table 1B).

Finally, mature *L. monocytogenes* biofilms were supplemented with PCM, 24 h after biofilm initiation. Biofilms were grown in TSB as described above, including the addition of fresh medium following the 3 h adhesion period, for a total of 24 h. At this time the spent medium was removed and the biofilms were washed twice with 25 ml of PBS, prior to the addition of 20 ml of either PAO1 PCM or *P. putida* PCM. Control biofilms were washed with PBS and supplemented with fresh TSB. The biofilms were then incubated for another 24 hours (Table 1C). All biofilms were incubated at either 22°C or 37°C with shaking at 90 rpm.

3.2.3.3 Enumeration of biofilm cells

Following incubation, the biofilms were analyzed by determining cell counts. Spent medium was removed and 25 ml of PBS was slowly pipetted into the Petri dishes to remove non-adherent cells from the steel coupons. Then, each coupon was placed vertically into a sterile tube where they were rinsed twice more by pipetting 10 ml of PBS into the tubes. Next, adherent cells were detached by vortexing for 3 min in 3 ml of PBS. The cell suspensions were serially diluted in PBS and enumerated by standard

plate counts using the drop-plate method. For each dilution, five 10 µl drops were dispensed on TSA and the colonies were enumerated after incubation for 24 h at 37°C.

3.2.4 Formation of biofilms on polystyrene

3.2.4.1 Biofilm growth and treatment with PCM

The effect of substances secreted by *Pseudomonas* spp. on *Listeria* biofilms on polystyrene was examined using the same approach described for stainless steel, by introducing either PAO1 PCM or *P. putida* PCM to the microtiter plates at different times after inoculation (0, 3, 24 h). *Listeria* biofilms were supplemented with PAO1 PCM or *P. putida* PCM at three stages of biofilm development: (1) initial cell attachment (2) growing biofilms and (3) mature biofilms, as outlined in Table 2.

L. monocytogenes biofilms were grown in sterile tissue culture treated 96-well plates (Costar 3596) using a modified version of previously published protocols (Borucki et al., 2003; Lemon et al., 2007). *Listeria* cultures were grown 24 h at 30°C on TSA plates from frozen stocks stored at -80°C. Single colonies were then swabbed over TSA plates and incubated overnight at 30°C. The bacterial lawns were scraped into sterile medium and dispersed using a vortex mixer. The resulting culture was then diluted to OD_{600} = 0.05 ± 0.01 (corresponding to 10^6 cfu/ml), and wells were inoculated with 150 µl of the standardized cell suspension. After 24 h, spent medium was removed and the biofilms washed with 150 µl of PBS. Fresh medium (150 µl) was then added to each well and the plates were incubated for an additional 24 h. All biofilms were incubated for a total of 48 h, with fresh medium added after 24 h. Each plate included 8 control wells containing sterile TSB.

To study the impact of treatment on biofilm formation, *Listeria* biofilms were prepared as described above using either PAO1 PCM or *P. putida* PCM throughout the entire incubation period, with fresh PCM added after 24 h (Table 2A).

To examine the impact on a growing biofilm, PCM was introduced 3 h after biofilm initiation in TSB. Following a 3 h adhesion period, the biofilms were washed once with 150 µl of PBS, and supplemented with 150 µl of either PAO1 PCM or *P. putida* PCM. Control biofilms were washed with PBS and supplemented with fresh TSB. The medium was replaced after 24 h of incubation (Table 2B).

To test the ability of PCM to disrupt mature biofilms, the biofilms were grown in TSB for 24 h prior to the introduction of the conditioned medium. After 24 h, the wells were washed once with 150 µl of PBS, and 150 µl of either PAO1 PCM or *P. putida* PCM was added. Control wells were washed once with PBS and supplemented with fresh TSB. The plates were then incubated for an additional 24 h (Table 2C). Biofilms were incubated statically at 22, 30, or 37°C for a total of 48 h, with at least 12 replicates for each test.

3.2.4.2 Quantification of biofilms on polystyrene

Following incubation, residual medium was removed using a multi-well pipettor and the wells were rinsed three times with 150 µl of sterile distilled water to remove loosely attached cells. The plates were dried in an inverted position for 30 minutes prior to staining with 50 µl of a 0.1% crystal violet solution for 45 minutes at room temperature. The plates were washed by rinsing 3 times with 150 µl of sterile distilled water and stain retained on the wells was solubilised by incubating for 30 min at 4°C

with 200 µl of 85% ethanol. The liquid was then transferred (150 µl) to a clean 96-well plate and the OD_{600} of the crystal violet was measured using a microtiter plate reader (FLUOstar Optima, BMG Labtech).

3.2.5 Statistical analysis

Data was analyzed using SigmaStat software (version 2.03). Mann Whitney tests were used to compare significant differences between treatments.

3.3 Results

3.3.1 Treatment with *P. putida* PCM

The effect of the *P. putida* PCM on *L. monocytogenes* biofilms was variable, depending on substrate material, incubation temperature, and the stage of biofilm development when the conditioned medium was introduced (Table 3).

3.3.1.1 Biofilms grown on stainless steel

At 22°C, length of development of the biofilm prior to exposure to *P. putida* PCM played a role. The biofilms were supplemented with *P. putida* PCM at three different stages of development. Biofilms grown entirely in the conditioned medium showed a slight reduction in adhesion, while treatment of a three hour biofilm did not affect adhesion. When a mature 24 h *Listeria* biofilm was supplemented with *P. putida* PCM a slight increase of 0.56 Log CFU/cm^2 over the control was observed (figure 1A).

At 37°C, *P. putida* PCM enhanced the growth of *L. monocytogenes* biofilms at all stages of growth (figure 1B). The greatest enhancement was observed for *L.*

monocytogenes biofilms grown entirely in the presence of *P. putida* PCM. This treatment produced a 0.78 Log CFU cm^{-2} increase in the number of adherent cells.

3.3.1.2 Biofilms grown on polystyrene

Tests conducted on polystyrene were carried out at 22, 30 and 37°C. In comparison to biofilms grown on stainless steel, results on polystyrene indicate that the properties of the substrate material affect the interaction between *L. monocytogenes* and *P. putida* PCM. At 22°C, as with biofilms grown on stainless steel, the effect depended on the stage of biofilm development when the PCM was introduced (figure 1C). Biofilms grown entirely in the presence of *P. putida* PCM were significantly reduced compared to the controls. However, when the *L. monocytogenes* biofilms were allowed to develop for 3 and 24 h prior to exposure to *P. putida* PCM, the decrease in adhesion was not as dramatic. Biofilms incubated at 30°C showed similar results, with significant inhibition of adhesion occurring for biofilms grown entirely in the presence of *P. putida* PCM, a minor decrease in adhesion of a 3 h biofilm, and no significant change in the mature biofilm (figure 1D). These results suggest that *P. putida* produces a substance that interferes with the initial adhesion of *L. monocytogenes* to polystyrene, but that is less efficient in dispersing a preformed biofilm.

L. monocytogenes was found to adhere poorly to polystyrene at 37°C, with little detectable biofilm remaining following the washing steps. Despite weak attachment at this temperature, biofilm formation was significantly enhanced with the addition of *P. putida* PCM (figure 2).

3.3.2 Treatment with *P. aeruginosa* PAO1 conditioned medium

PAO1 PCM modulated the growth of *L. monocytogenes* biofilms in a more consistent pattern than was observed with *P. putida* PCM. Although results did vary depending on incubation temperature, similar outcomes were obtained regardless of the substrate material used or the stage of biofilm growth at the time of treatment (Table 3).

3.3.2.1 Biofilms grown on stainless steel

On stainless steel, the effect of PAO1 PCM on *L. monocytogenes* biofilms depended on incubation temperature. When incubated at 22°C, exposure to PAO1 PCM reduced the number of adherent cells by up to 1.65 Log CFU/cm^2 (figure 3A). This reduction was observed regardless of the stage of development when the biofilms were exposed to the medium, indicating that PAO1 PCM secretes a substance that is capable of both preventing the formation of *L. monocytogenes* biofilms as well as dispersing mature, preformed biofilms. In contrast, at 37°C treatment with PAO1 PCM did not produce a significant change in the cell counts of *L. monocytogenes*, with the exception of the 3 h biofilm which showed a slight increase in adhesion (figure 3B).

3.3.2.2 Biofilms grown on polystyrene

There did not seem to be an effect when PAO1 PCM was introduced to the biofilms at 37°C (data not shown). As *Listeria* did not produce stable biofilms on polystyrene at this temperature, any decrease in adhesion would not be detectable, and treatment with PCM did not enhance adhesion. However, at 22°C PAO1 PCM significantly inhibited the growth of *L. monocytogenes* biofilms at all stages of

maturation. Although the trend was the same as on steel, the effect was more dramatic on polystyrene where treatment eliminated virtually all detectable biofilm. PAO1 PCM both prevented the formation of *L. monocytogenes* biofilms when added during the early stages of development, and eliminated the mature biofilms when added after 24 h of growth (figure 3C). Similar results were observed at 30°C, where treatment with PAO1 PCM significantly inhibited biofilm formation at all stages of growth (figure 3D).

3.4 Discussion

Numerous studies have investigated the growth of *Listeria* in mixed culture biofilms, and there have been conflicting reports of both enhancement and reduction of growth. For example, Guobjornsdottir et al. (2005) reported that *L. monocytogenes* grown with *P. fluorescens* have a lower rate of attachment to stainless steel, while a culture of mixed *Pseudomonas* spp. significantly enhances biofilm growth. Similarly, when Carpentier and Chassaing (2004) investigated the growth of *L. monocytogenes* biofilms in mixed cultures with a range of organisms isolated from a food processing environment, two strains of *P. fluorescens* were found to decrease attachment of *Listeria* to steel and one strain had no effect, while five strains of *P. putida* decreased attachment and a sixth had no effect.

This type of variation in the impact on *Listeria* biofilms can also be seen when a single strain of *Pseudomonas* sp. is tested under varying environmental conditions. For example, Buchanan and Bagi (1999) examined the effect of a single strain of *P. fluorescens* on the growth of *L. monocytogenes* under varying temperature, pH, and sodium concentrations and found that, depending on the culturing conditions, *P.*

fluorescens could enhance, suppress, or have no effect on the growth of *L. monocytogenes*. Similar disparities were observed in this study when culture supernatants of single strains of *P. putida* and *P. aeruginosa* PAO1 were tested (Table 3). As was pointed out by Buchanan and Bragi (1999), this type of result may explain the numerous and conflicting reports regarding the impact of *Pseudomonas* spp. on the growth of *Listeria*.

In this study, biofilms of *L. monocytogenes* were treated with filtered supernatants from *P. putida* and *P. aeruginosa* cultures to determine whether these species secrete substances capable of modifying *Listeria* biofilms. It has been suggested that *Listeria* requires the cells and EPS of a primary colonizer, particularly *Pseudomonas* spp., to enhance its biofilm forming ability (Chavant et al., 2002; Sasahara & Zottola, 1993). The results of this study demonstrate that substances secreted by *Pseudomonas* spp. into the medium also modulate *Listeria* biofilm formation, and that incubation temperature, substrate properties, and the stage of biofilm development at the time of exposure to these substances will determine the manner in which biofilm growth is altered.

3.4.1 Effect of temperature

Although a reduction was observed when the biofilms were supplemented with PAO1 PCM during all stages of biofilm development and on both surface materials tested, the effect was temperature dependent. Treatment with PAO1 PCM clearly reduced biofilm formation at 22 and 30°C; however, treatment did not affect biofilms grown at 37°C. Temperature also affected how the biofilms responded to treatment with *P. putida* PCM, where increases were consistently observed for biofilms grown at 37°C,

while at 22 and 30°C the outcome varied with substrate material and the stage of biofilm development.

The role of incubation temperature in how the conditioned medium altered *L. monocytogenes* biofilms likely reflects the temperature-dependent physiological changes known to occur in *Listeria*. Characteristics such as cell surface hydrophobicity (Chavant et al., 2002), membrane composition (Gandhi & Chikindas, 2007), and flagellation and motility (Grundling et al., 2004; Shen & Higgins, 2006) are all affected by incubation temperature. Notably, *Listeria* is motile and produces peritrichous flagella when grown at 30°C and below, whereas transcription of flagella is repressed at 37°C (Shen & Higgins, 2006; Shen et al., 2006).

Lemon et al. (2007) demonstrated that flagellar motility is critical to biofilm formation by *L. monocytogenes*, during both the initial attachment stages as well as the subsequent biofilm growth. This observation hints towards two possible mechanisms for how *P. aeruginosa* PCM reduces biofilm formation, as this reduction was only observed at temperatures amenable to flagella expression. The conditioned medium could alter biofilm formation by either interfering with the expression of flagella, or by interfering with the motility of the flagella, as it has been shown that flagella-minus and paralyzed-flagellum mutants have comparable defects in biofilm formation (Lemon et al., 2007).

The finding that flagella are essential to both the initiation and subsequent development of *L. monocytogenes* biofilms (Lemon et al., 2007) fits with the hypothesis that *P. aeruginosa* PCM modifies either the presence or motility of *Listeria*'s flagella and the observation that treatment with conditioned medium alters biofilm formation at all stages of growth.

Furthermore, Lemon et al. (2007) found that the defect in adhesion to stainless steel by non-motile mutants was less pronounced than their defect in adhesion to glass, and propose that the role of motility may be more important on some surfaces than others. Similarly, Chavant et al. (2002) found that *L. monocytogenes* could produce stable biofilms on PTFE when grown at 20°C, but not at 37°C, suggesting that flagella facilitate contact with the surface by overcoming electrostatic repulsion forces. These findings are compatible with observations made in this study, where a greater reduction in biofilm formation was observed on polystyrene than on stainless steel. This may also explain why at 37°C, when expression of flagella is repressed, stable control biofilms could be produced on stainless steel, but not on polystyrene.

The effect of *P. putida* PCM on the attachment of *Listeria* may also involve the flagella. It is possible that *P. putida* may secrete a substance that compensates in some way for *Listeria*'s lack of motility at high temperatures, as attachment was enhanced after treatment with *P. putida* PCM at 37°C, when *L. monocytogenes* is not flagellated. Biofilms grown on stainless steel at 37°C that were exposed to *P. putida* PCM had a similar level of attachment as the flagellated control biofilms grown at 22°C. Similarly, at 37°C on polystyrene, where only minimal amounts of attached cells could be detected for biofilms grown in TSB, supplementation with *P. putida* PCM doubled adhesion of *Listeria*.

3.4.2 Effect of substrate

L. monocytogenes biofilms were grown on two common food contact surfaces with different physicochemical properties, stainless steel 316 (hydrophilic) and polystyrene (hydrophobic) (Palmer et al., 2007; Tresse et al., 2007).

In general, the physicochemical properties of the support and the bacterial cell surface are considered to affect the attachment of cells, as biofilm formation involves van der Waals and electrostatic forces and hydrophobic interactions (Palmer et al., 2007; Planchon et al., 2006; Tresse et al., 2007). It has also been found that the proteinaceous complexes and cellular features formed by attached bacteria can differ depending on the nature of the substrate (Cheung et al., 2007). Despite this, pure cultures of *L. monocytogenes* have been reported to adhere to stainless steel and polystyrene in similar numbers (Tresse et al., 2007), and *Listeria* has been shown to attach to a variety of materials of differing hydrophobicities representing metals, rubbers, and polymers (Beresford et al., 2001). Based on these observations, it has been concluded that the ability of *Listeria* to adhere to an inert surface is stronger than the influence of the physicochemical surface properties (Tresse et al., 2007). However, Chavant et al. (2002) found that the presence of similar initial populations does not imply the same kinetics of colonization on different surfaces, and the properties of the substrate material do appear to play a role in how *L. monocytogenes* biofilms respond to treatment with *Pseudomonas* conditioned medium.

Treatment of the biofilms with *P. putida* PCM on stainless steel had little effect at 22°C, whereas those grown on polystyrene showed a more consistent reduction in adhesion. In contrast, treatment with PAO1 PCM had the same effect on both materials, decreasing the numbers of attached cells, although the magnitude of this decrease was greater on polystyrene where the biofilms were virtually eliminated. These findings suggest that attachment to polystyrene may be weaker and more easily interrupted than on stainless steel. This may be related to the hydrophobic interactions between the

hydrophobic polystyrene surface and the cell surface of *L. monocytogenes*, which has been shown to be hydrophilic (Mafu et al., 1991).

In the case of the control, biofilms produced using *L. monocytogenes* alone, biofilm development on polystyrene at 37°C was minimal, whereas on stainless steel cell counts were lower at 37°C than 22°C, but the difference was not statistically significant. Chavant et al. (2002) noted that significant detachment occurs when *L. monocytogenes* biofilms are grown on PTFE at 37°C due to weak interactions between the cells and the surface, and a similar scenario may apply to the polystyrene used in this study.

Biofilms prepared on steel were inoculated with high density culture (10^8 cfu/ml) and incubated for a 3 h adhesion period before removing planktonic cells, whereas biofilms prepared on polystyrene were inoculated with a low density culture (10^6 cfu/ml) for 24 h prior to washing. This discrepancy in the methods used to prepare biofilms on steel and polystyrene cannot be discounted as a factor in the observed differences between the two substrates. However, preliminary tests with biofilms grown on polystyrene that included a high inoculum and a 3 h adhesion period did not produce significantly different results from those that used a lower inoculum without washing (data not shown). A protocol that used low density inoculum was chosen to remain consistent with previously published studies using microtiter plates (Borucki et al., 2003; Irie et al., 2005; Lemon et al., 2007; Valle et al., 2006).

3.4.3 Effect of stage of biofilm development

To examine how *Pseudomonas* conditioned medium (PCM) affect *L. monocytogenes* biofilms at different stages of maturation, the biofilms were exposed to

Pseudomonas conditioned medium after 0, 3, and 24 hours of development. This approach enabled observation of any alterations in biofilm initiation and growth, and the impact on mature biofilms.

Biofilm formation is considered a well regulated developmental process, consisting of discrete stages (reversible attachment, irreversible attachment, maturation, dispersal) (O'Toole et al., 2000; Sauer et al., 2002), each of which have distinct physiological characteristics (Sauer et al., 2002). For example, Sauer (2002) found that protein expression in planktonic *P. aeruginosa* was more similar to that of *P. putida* than to *P. aeruginosa* in the maturation stage of biofilm development. They also demonstrated that *P. aeruginosa* displays multiple phenotypes and stage-specific physiology during biofilm development. The temporal expression of biofilm specific genes has also been demonstrated in *P. aeruginosa* (Southey-Pillig et al., 2005), and although fewer studies have been conducted using *L. monocytogenes*, gene expression has been demonstrated to vary with the stage of biofilm development (Rieu et al., 2007).

The significance of the stage of biofilm development on the impact of PCM was most evident when *P. putida* PCM was applied to *L. monocytogenes* biofilms grown on polystyrene at 22 and 30°C. When added at time 0, *P. putida* PCM significantly inhibited biofilm formation. When added after 3 and 24 h of biofilm development, the *P. putida* PCM also reduced the amount of biofilm formed, but this was not always statistically significant. On stainless steel, *P. putida* PCM enhanced growth at all stages of biofilm development at 37°C, with slightly greater enhancement when the conditioned medium was added during the early stages of development.

Other studies have also found that the stage of maturation may lead to differential responses to exposure to conditioned medium. Valle et al., (2006) reported that spent supernatant from the uropathogenic *E. coli* strain CFT073 prevented initial biofilm formation of the commensal *E. coli* strain MG1655 F' and blocked further development of biofilms when added at 0, 1, 3, and 6 hours after biofilm initiation; however, the supernatant did not induce dispersal of mature pre-formed biofilms. It is possible that the effect of *P. putida* PCM in this study may be similar to that observed by Valle et al. (2006) for *E. coli* CFT073, where treatment is slowing additional growth of the biofilm. However, as there is no reduction in growth for biofilms on stainless steel at any stage of biofilm development, it appears more likely that that the conditioned medium only interferes with the initial stages of attachment of *Listeria* on polystyrene.

Treatment with *P. aeruginosa* PAO1 PCM reduced biofilm growth at all stages of biofilm development below 37°C. *P aeruginosa* secretes a substance that is capable of halting biofilm formation, as well as disrupting preformed biofilms on both stainless steel and polystyrene. Irie et al. (2005) reported similar results using the spent supernatant from *P. aeruginosa* PAO1, which was found to disrupt pre-formed biofilms of *B. bronchiseptica* after four hours of exposure, and after 10 minutes under conditions of high mechanical sheer.

3.5 Conclusion

This study demonstrates that substances secreted by *Pseudomonas* spp. are capable of modulating the growth of *L. monocytogenes* biofilms. Depending on the species and environmental conditions, the outcome may be either an increase or a decrease in biofilm growth.

The strong influence of culturing conditions on the effect of treatment with *P. putida* conditioned medium suggests the possibility that a number of different compounds are produced which have varied effects depending on the environment. It also illustrates the importance of environmental factors in the interactions between organisms in a food processing setting.

The observed reduction in attachment and disruption of *L. monocytogenes* biofilms when treated with *P. aeruginosa* PAO1 conditioned medium suggests that this bacterium secretes a substance that could be used as part of an antimicrobial strategy. Other studies have investigated the use of substances produced by microorganisms as a method to prevent the formation of *Listeria* biofilms. For example, Leriche and Carpentier (2000) found that *Staphylococcus sciuri* biofilms significantly reduced the adhesion of *L. monocytogenes* to stainless steel. They hypothesize that the decreased adhesion may involve the properties of the EPS, and suggest the use of *S. scuri* biofilms to enhance the fight against *Listeria* in food industry premises. Similarly, Meylheuc *et al.* (2006) found that preconditioning stainless steel with biosurfactants produced by *Pseudomonas fluorescens* and *Lactobacillus helveticus* reduced adhesion of *L. monocytogenes* by 75.2 and 99.7%, respectively, and suggest conditioning solid surfaces in industrial plants to prevent contamination by *Listeria*.

The consistent reduction in biofilm formation as a result of treatment with *P. aeruginosa* PAO1 PCM may indicate that a single secreted substance or group of substances is responsible for the reduction. A notable candidate would be the biosurfactant rhamnolipid, which Irie et al. (2005) identified as the substance in *P. aeruginosa* PAO1 conditioned medium responsible for the disruption *B. bronchiseptica*

biofilms. Rhamnolipids possess anti-adhesive activity against a variety of bacteria and yeasts (Rodrigues et al., 2006), and have been proposed to reduce adhesion of planktonic cells to preformed biofilms in addition to accelerating the dispersion phase of biofilm development (Davey et al., 2003; Irie et al., 2005). The temperature dependent manner in which the *P. aeruginosa* PAO1 PCM disrupted *L. monocytogenes* biofilms implies that one or several substances in the PAO1 PCM affected the flagella of the *Listeria* cells. If the conditioned medium is altering the function of *Listeria* flagella, it may be doing so via a physical modification of the flagella or through a cell-signalling mechanism and identification of the substance responsible may give clues to the mechanisms behind the observed reduction in biofilm formation.

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		Treatment		
	Time (h)	**PAO1 PCM**	***P. putida* PCM**	**Control**
A.	0	PCM	PCM	TSB
	3	PCM	PCM	TSB
	24	Analyze	Analyze	Analyze
B.	0	TSB	TSB	TSB
	3	PCM	PCM	TSB
	24	Analyze	Analyze	Analyze
C.	0	TSB	TSB	TSB
	3	TSB	TSB	TSB
	24	PCM	PCM	TSB
	48	Analyze	Analyze	Analyze

Table 1. Treatment of *Listeria* biofilms grown of stainless steel

(A) Biofilms exposed to PCM at the beginning of biofilm initiation; (B) biofilms exposed to PCM 3 h after biofilm initiation; (C) mature biofilms exposed to PCM 24 h after biofilm initiation, for an additional 24 h.

		Treatment		
	Time (h)	**PAO1 PCM**	***P. putida* PCM**	**Control**
A.	0	PCM	PCM	TSB
	24	PCM	PCM	TSB
	48	Analyze	Analyze	Analyze
B.	0	TSB	TSB	TSB
	3	PCM	PCM	TSB
	24	PCM	PCM	TSB
	48	Analyze	Analyze	Analyze
C.	0	TSB	TSB	TSB
	24	PCM	PCM	TSB
	48	Analyze	Analyze	Analyze

Table 2. Treatment of *Listeria* biofilms grown on polystyrene

(A) Biofilms exposed to PCM at the beginning of biofilm initiation; (B) biofilms exposed to PCM 3 h after biofilm initiation; (C) mature biofilms exposed to PCM 24 h after biofilm initiation, for an additional 24 h.

A.

Temperature	22°C			30°C			37°C		
Length of growth before addition of *P. putida* PCM (h)	0	3	24	0	3	24	0	3	24
Steel	↓	↔	↔	nd	nd	nd	↑	↑	↑
Polystyrene	↓	↓	↓	↓	↓	↔	↑	↑	nd

B.

Temperature	22°C			30°C			37°C		
Length of growth before addition of PAO1 PCM (h)	0	3	24	0	3	24	0	3	24
Steel	↓	↓	↓	nd	nd	nd	↔	↑	↔
Polystyrene	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	↔	nd	nd

nd =no data

Table 3. Summary of results

Arrows represent a statistically significant difference from controls ($p < 0.05$). (A) *Listeria* biofilms treated with *P. putida* PCM; (B) *Listeria* biofilms treated with PAO1 PCM.

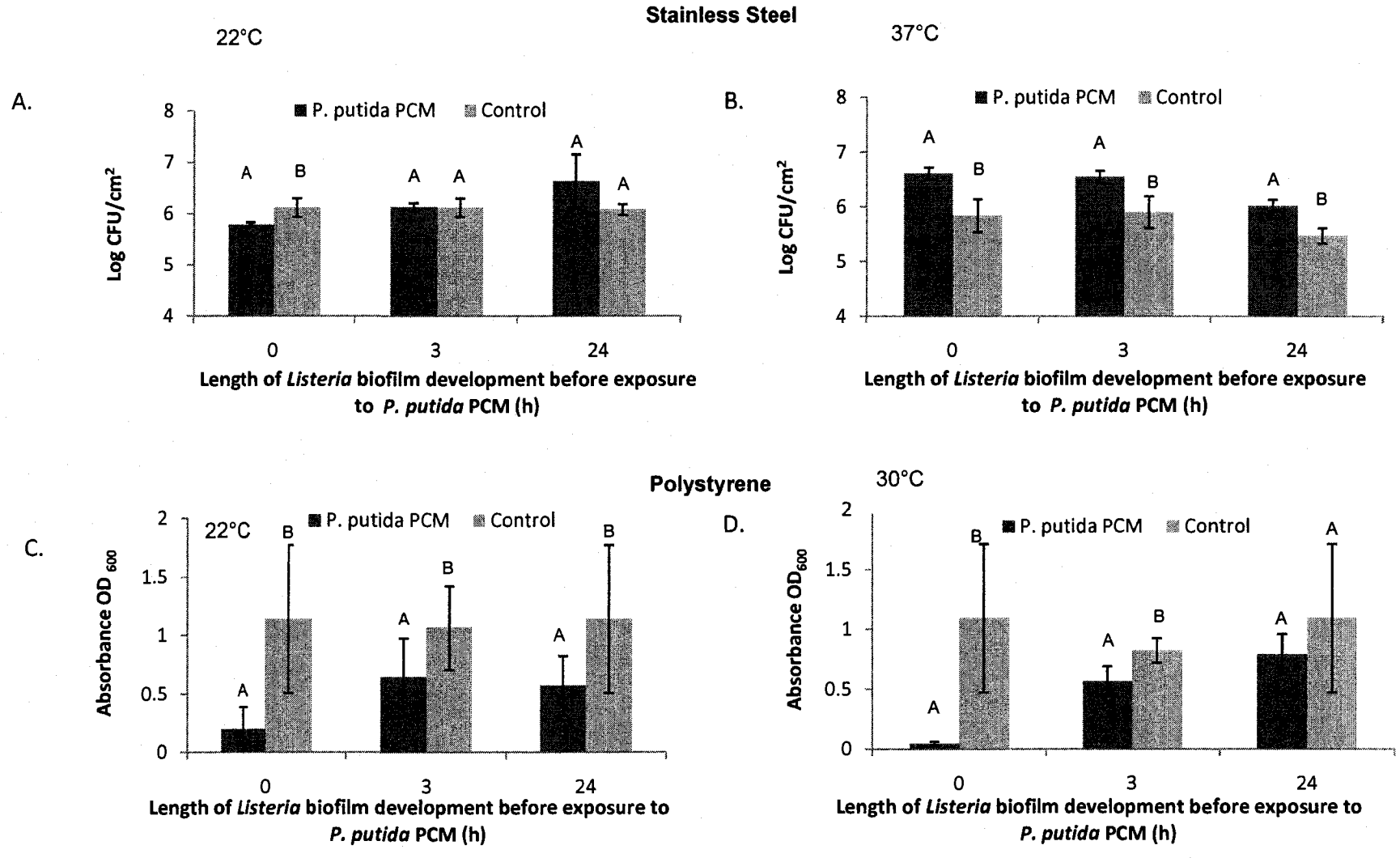

Figure 1. Effect of *P. putida* PCM on *Listeria* biofilms

(A) Biofilms on steel, incubated at 22°C (B) Biofilms on steel, incubated at 37°C (C) Biofilms on polystyrene, incubated at 22°C (D) Biofilms on polystyrene, incubated at 30°C. For each treatment, bars with the same letter are not significantly different from each other ($p < 0.05$).

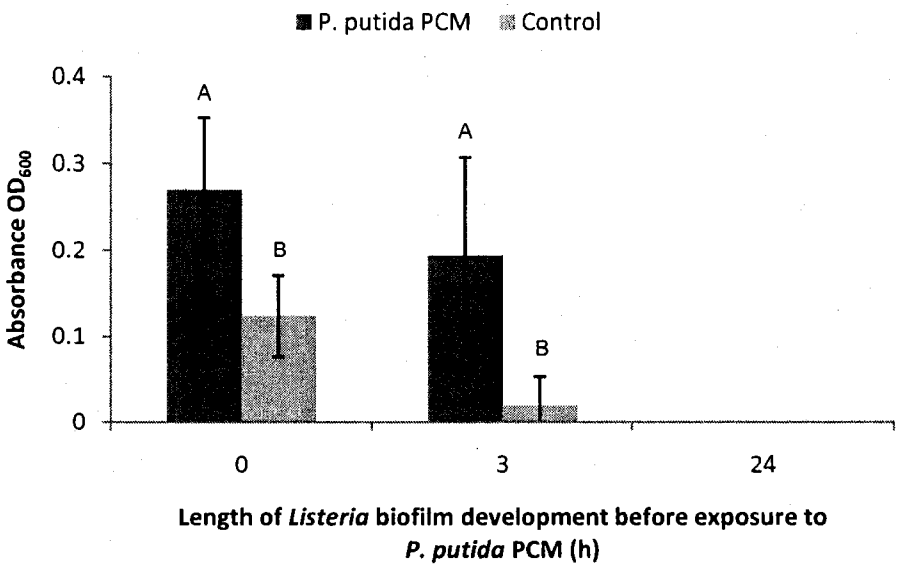

Figure 2. Effect of *P. putida* PCM on *Listeria* biofilms on polystyrene at 37°C

L. monocytogenes exhibited poor adhesion under these conditions; however treatment with *P. putida* PCM significantly enhanced attachment. Tests were limited to 0 and 3 h *Listeria* biofilms. For each treatment, bars with the same letter are not significantly different from each other ($p < 0.05$).

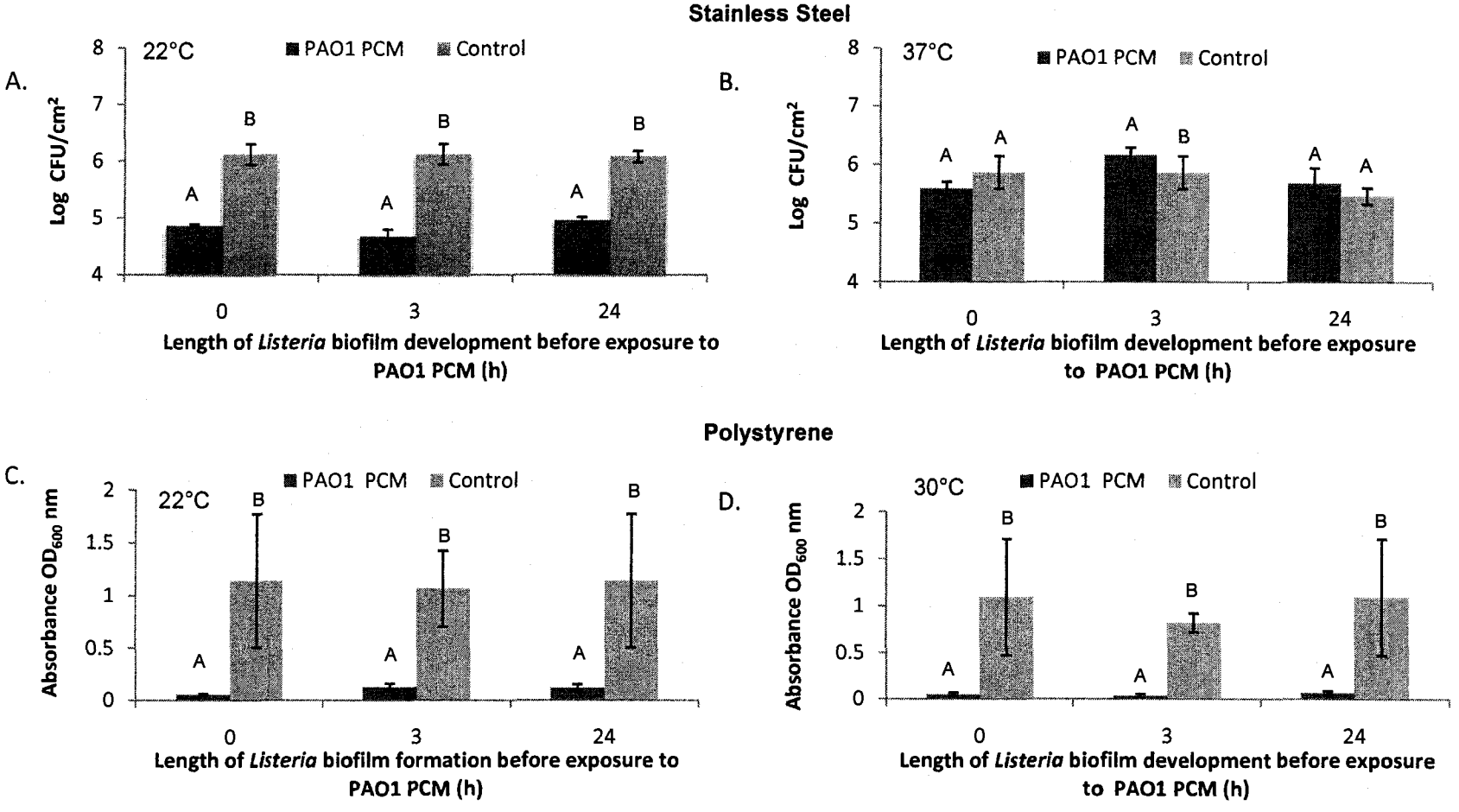

Figure 3. Effect of PAO1 PCM on *Listeria* biofilms

(A) Biofilms on steel, incubated at 22°C (B) Biofilms on steel, incubated at 37°C (C) Biofilms on polystyrene, incubated at 22°C (D) Biofilms on polystyrene, incubated at 30°C. For each treatment, bars with the same letter are not significantly different from each other ($p < 0.05$)

4. Identification of *Pseudomonas aeruginosa* PAO1 genes involved in inhibiting the growth of *Listeria monocytogenes* biofilms by transposon mutagenesis

4.1 Introduction

Mobile genetic elements are segments of DNA that can relocate between genomic sites, such as transposons, insertion sequences, pathogenicity islands, and plasmids (Hayes, 2003; Nagy & Chandler, 2004). They are ubiquitous in nature, and have been found in Eubacteria, Archaea, and Eukarya (Hayes, 2003). DNA transposition is a biological phenomenon that provides adaptive advantages such as genetic plasticity, inheritance of antibiotic resistance, and integration of retroviral DNA (Goryshin et al., 2000), and has also proven to be an important tool in bacterial genetic research.

Transposon mutagenesis has been used to study a variety of yeasts and bacteria, including both Gram negative and Gram positive species (Goryshin et al., 2000; Mormann et al., 2006). Furthermore, it has also been shown to be an effective technique even in slow-growing, fastidious species such as *Bartonella* sp., and in species with large genomes such as *Pseudomonas* spp. (Filiatrault et al., 2006; Hoffman et al., 2000). Integration of a transposon into the genome may result in gene knockouts or altered expression of nearby genes, producing phenotypic changes in the host organism and enabling the study of non-essential genes (Hoffman et al., 2000). This approach has been used to produce near-saturation libraries of a number of

species, including *Neisseria* sp., *Mycoplasma* sp., and *Pseudomonas aeruginosa* (Geoffroy et al., 2003; Hutchison et al., 1999; Jacobs et al., 2003).

Conventional studies using random mutagenesis produce knockout mutant libraries, from which individual mutants are then screened for a phenotype of interest based on negative selection. More complex uses of transposons include techniques such as signature-tagged mutagenesis (STM), a negative selection method used to study genes in a pathogen that are required for host colonization, in which the transposons contain sequence tags that can be amplified by PCR (Hayes, 2003; Saenz & Dehio, 2005; Salama & Manoil, 2006).

Transposons containing reporter genes such as *lux*CDABE, *lacZ*, and *phoA* have been used as a tool to investigate gene expression (Hayes, 2003; Lewenza et al., 2005). Screening of random mutants has also been paired with genomic microarrays to enable analysis of large numbers of mutants (Salama & Manoil, 2006; Winterberg et al., 2005).

Transposon mutagenesis provides a practical approach to analyze genes of unknown function and unknown bacterial pathogenicity factors, and the transposons that are most commonly chosen as genetic tools are those that insert randomly, such as the Tn5 (Hayes, 2003; Riess et al., 2003; Saenz & Dehio, 2005). Tn5 DNA transposition requires just three macromolecules: the transposon, target DNA, and transposase (Tnp) which catalyses the reaction (Reznikoff, 2003). The traditional view of theTn5 transposon consists of two inversely oriented insertion sequences (IS) (IS*50* elements), each containing two 19 bp sequences that are critical Tnp binding sites, separated by resistance genes for kanamycin, bleomycin, and streptomycin (Hayes, 2003). The gene

coding for Tnp is on the IS*50* element. However, as long as there is a source of Tnp, a simplified transposon can be produced consisting of the specific 19 bp sequences on either end of virtually any DNA fragment (Reznikoff, 2003).

Transposition proceeds via a cut and paste mechanism that involves the following steps: (1) Tnp binds the ends of the transposon together in a synaptic complex; (2) DNA cleavage to release the transposon from its donor site; (3) binding to target DNA, to join the transposon ends to the new site; (4) removal of transposase and repair of DNA gaps at the insertion site by host factors (Goryshin et al., 2000; Hayes, 2003; Reznikoff, 2003). The wild-type Tn5 transposase is a relatively inactive protein, as regulation of transposition activity is necessary to balance adaptive advantages with potentially lethal DNA rearrangements (Gueguen et al., 2005). This inactivity is due in part to poor binding to the 19 bp end sequences, however, a hyperactive mosaic version of the 19 bp end sequence has been identified (mosaic ends), and this enhanced Tnp activity enables the use of Tn5 transposons as an effective tool for genetic analysis (Reznikoff, 2003).

One approach to transposition involves the introduction of the transposon into the host on a suicide vector, with expression of transposase within the host in subsequent generations. Alternatively, a stable Tn5 transposition complex called a Transposome, consisting of a transposon and purified transposase in the absence of Mg^{++}, can be produced in vitro and electroporated into the target cell (Goryshin et al., 2000). The Transposome undergoes transposition in the presence of target DNA and Mg^{++}, which are required for the insertion of the transposon, and allows for in vivo insertion of the transposon into the chromosome (Hoffman et al., 2000).

Random mutagenesis using Transposomes has been carried out in a variety of bacterial species to create knockout mutants (Filiatrault et al., 2006; Goryshin et al., 2000; Hoffman & Jendrisak, 2002; Hoffman et al., 2000; Riess et al., 2003). In this study, Transposomes were used to randomly mutate *P. aeruginosa* PAO1 to gain insight into the genetic basis of its ability to remove *Listeria* biofilms, and screening of the mutants revealed that the secreted protease elastase is involved.

P. aeruginosa secretes a variety of substances into the extracellular environment, including virulence factors such as ExoS, ExoT, ExoU and ExoY, as well as many other proteins, and at least four proteases: elastase, LasA, alkaline protease, and a lysine-specific endopeptidase called protease IV (Braun et al., 2001; Cowell et al., 2003; Kessler et al., 1998; Malloy et al., 2005). The most abundant secreted protein is elastase, a 33 kDa zinc metalloprotease encoded by the *lasB* gene (Braun et al., 2001; Kessler et al., 1998).

Besides being the predominant secreted protein, elastase is also the most potent of the *P. aeruginosa* proteases, and it is able to cleave many proteins at multiple sites (Kessler et al., 1998). It has been demonstrated to degrade or inactivate a range of host tissues, immune system components, and proteases (Rust et al., 1996). In addition to exhibiting elastolytic activity, elastase also degrades collagen and fibrin, as well as immunoglobulins, serum complement factors, MMP2 AND MMP9, and the cytokines gamma interferon and tumor necrosis factor alpha (Cowell et al., 2003; Kessler et al., 1998; Rust et al., 1996). The major role of *P. aeruginosa* proteases during infection is thought to involve tissue penetration, and elastase has been reported to be involved in the regulation of invasion (Cowell et al., 2003).

Beyond its functions during infection, a number of other roles have been reported for elastase. For example, it has been demonstrated to be essential for normal biofilm development, and mutants lacking elastase produce biofilms with about 50% less biomass (Overhage et al., 2008). It is also critical to swarming motility, a multicellular phenomenon that involves the movement of bacterial populations across a semi-solid surface and is characterized substantial changes in gene expression (Overhage et al., 2008; Overhage et al., 2007). Furthermore, it is responsible for processing secreted proenzymes, such as pro-LasA and pro-LasD, to convert them to their active form (Braun et al., 1998; Kessler et al., 1998).

Elastase itself is produced as a proenzyme, and is secreted by the type II secretion system. Secretion of enzymes synthesized in the cytoplasm of Gram negative species involves passing through both membranes of the cell envelope, and six major mechanisms for protein transport have been identified (Bingle et al., 2008). *P. aeruginosa* uses a number of these pathways for the secretion of proteins, including types I, II, and III, the autotransporter pathway (also known as the type V system), as well as the most recently identified pathway, the type VI secretion system (Bingle et al., 2008; Braun et al., 2001; Henderson et al., 2004). Along with elastase, the majority of *P. aeruginosa* exoproteins are secreted by the type II pathway, including lipase, alkaline phosphatase, exotoxin A, LasA among others (Braun et al., 2000b). The type II pathway is a two step process that involves translocation across the inner membrane by the Sec machinery and subsequent secretion across the outer membrane via a complex consisting of at 12 proteins encoded by the *xcp* genes (Henderson et al., 2004).

The synthesis of elastase as a pre-proenzyme, consisting of a signal peptide, a propeptide, and the enzyme, facilitates its transport across the cell envelope via the type II pathway. First, the N-terminal signal peptide targets the protein for translocation from the cytoplasm into the periplasm by the Sec secretion system, and is removed in the process (Braun et al., 2001; Kessler et al., 1998; McIver et al., 2004). In the periplasm, the propeptide serves as an intramolecular chaperone required for folding, and after further processing by autoproteolytic cleavage, the propeptide remains noncovalently associated with mature elastase as an inhibitor. The inactive propeptide-enzyme complex is then secreted by the Xcp complex to the extracellular space, where the propeptide dissociates and is degraded, presumably by elastase itself (Bingle et al., 2008; Braun et al., 2000b; McIver et al., 2004). Dissociation of the propeptide requires a host specific factor, which has been hypothesized to be part of the Xcp secretion machinery (Braun et al., 2001).

Pseudomonas putida does not produce elastase, and attempts to express *P. aeruginosa* elastase in *P. putida* did not result in secretion of the active enzyme (Braun et al., 2000a). As elastase is the most abundant of the proteins secreted by *P. aeruginosa*, it would be a principal component of PAO1 conditioned medium, and therefore it is likely that the composition of PAO1 conditioned medium would vary considerably from that of *P. putida*. This is consistent with our finding that *P. aeruginosa* conditioned medium inhibits *Listeria* biofilms, while treatment *P. putida* conditioned medium does not produce the same effect. In this study, we use random mutagenesis to demonstrate that *P. aeruginosa* elastase is involved in the removal of *Listeria* biofilms.

4.2 Materials and Methods

4.2.1 Optimizing growth of *L. monocytogenes* biofilms on polystyrene

To optimize the growth of *L. monocytogenes* biofilms on polystyrene, biofilms were grown in sterile tissue culture treated 96-well plates (Costar 3596) using inoculum grown at either 22 or 30°C. To prepare the inoculum, *L. monocytogenes* cultures (strain EGD) were grown for 24 h on TSA plates from frozen stocks stored at -80°C and single colonies were then swabbed over TSA plates and incubated overnight at either 22 or 30°C. The bacterial lawns were scraped into sterile TSB and dispersed using a vortex mixer and the resulting culture was then diluted to OD_{600} = 0.05 ± 0.01 (corresponding to 10^6 cfu/ ml).

These standardized cell suspensions (150 µl) were used to inoculate five eight-well rows in a 96-well microtiter plate, and experiments were carried out in triplicate for each inoculum growth temperature. Empty wells were filled with TSB to minimize evaporation. The plates were sealed with parafilm and incubated at 30°C for 24 h. Planktonic cells were then removed by washing with 150 µl of PBS using a multi-well pipettor, and 150 µl of fresh TSB was deposited onto the adherent bacteria. The plates were then sealed with parafilm and incubated for an additional 24 h. Subsequently, the biofilms were washed and stained with crystal violet as described in chapter 3 (section 3.2.4.2). To assess the amount of variation among the biofilms, a one-way ANOVA ($p < 0.05$) was performed to compare individual rows within a single plate and a second test was performed to evaluate the variance between plates.

4.2.2 Construction of *P. aeruginosa* PAO1 Tn mutants

P. aeruginosa PAO1 was subjected to insertional mutagenesis using Transposomes prepared with the EZ:Tn5 <Tet-1> Insertion Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions. Briefly, Transposomes were formed by combining 2 µl of the Tn5 transposon with 4 µl of transposase and 2 µl of 100% glycerol. The mixture was vortexed at maximum speed and incubated at room temperature for 30 minutes. Electroporation and preparation of electrocompetent *P. aeruginosa* cells using a microcentrifuge based method with 300 mM sucrose were carried out as described by Choi et al (2006). The electrocompetent cells and 1 µl of the Transposome mixture were transferred to a 2.0 mm gap electroporation cuvette (Eppendorf) and electroporated at 2500 volts (Eppendorf Electroporator 2510). Transformed cells were selected by plating on Luria-Bertani agar (LB; Becton Dickinson BBL) supplemented with 100 µg/ml tetracycline. After incubation at 37°C for 18h, individual resistant colonies were transferred with sterile toothpicks to 96-well microtiter plates containing 150 µl of LB medium supplemented with 10% glycerol. Plates were sealed with parafilm, incubated for 18h at 37°C, then frozen and stored at -80°C. A total of 1416 mutants were obtained. Insertion of the Tet-1 transposon into the chromosome was confirmed by PCR in five randomly selected mutants (forward, 5'-TATTTCTAGATTTCAGTGCAATTTA-3'; reverse, 5'-ACTCTAGAGGATCATGCGCACCC-3') using the following conditions: denaturation at 95°C for 3 min, annealing at 55°C for 30 s, and elongation at 72°C for 3 min for a total of 35 cycles.

4.2.3 Screening Tn insertion mutants

To elucidate the genetic basis of the antibiofilm effect, we tested the supernatant activity of the insertion mutants for an impaired ability to remove 24 h *Listeria* biofilms grown in polystyrene plates (Fig. 4). Tn mutants were incubated at 37°C for 48 h in 96-well 0.22 µm filter plates (Millipore Multiscreen HTS) containing 250 µl of TSB per well. Sterile supernatant from each of the mutants (mutant PCM) was obtained by centrifugation at 1000 x g for 10 minutes. Mutant PCM (~30 µl) was mixed with 100 µl of fresh TSB and used to treat *L. monocytogenes* biofilms (Valle et al., 2006).

The *L. monocytogenes* biofilms were prepared as described above (section 4.2.1) using inoculum prepared at 30°C. After incubation for 24 h at 30°C, spent medium and planktonic cells were removed using a multi-channel pipette and the biofilms were washed once with 150 µl of PBS. Biofilms were then incubated for an additional 24 h at 30°C in the presence of mutant PCM. Negative controls were incubated with TSB alone. Following incubation for an additional 24 h at 30°C, the biofilms were stained with crystal violet. Wells that retained the crystal violet stain were determined by visual inspection. Seven mutants produced supernatants that failed to remove adherent *L. monocytogenes* cells and were singled out for additional tests.

First, the screening process was repeated as described above with eight replications for each mutant. The mutants were then used to prepare larger volumes of *Pseudomonas* conditioned medium (PCM) as described in chapter three (section 3.2.2), by combining sterile mutant PCM with 2X concentrated TSB. *Listeria* biofilms were grown in 96-well plates for 24 h at 30°C, washed once with PBS and supplemented with 150 µl of mutant PCM or 150 µl of fresh TSB (controls). The plates were then incubated

for an additional 24 h, stained with crystal violet and biofilms quantified using a microtiter plate reader.

4.2.4 Characterization of Tn mutant 5B9

P. aeruginosa mutant 5B9 demonstrated impaired biofilm removal abilities and was selected for additional studies. Insertion of the Tet-1 transposon was confirmed by PCR using the conditions described above. Mutant 5B9 was found to be Gram negative and oxidase positive.

To analyze the growth of mutant 5B9, its growth curve was compared to the wild-type *P. aeruginosa* PAO1. Overnight cultures of mutant 5B9 and *P. aeruginosa* were set to an OD_{600} of 0.05 in LB (corresponding to 10^6 CFU ml^{-1} for both mutant 5B9 and the wild type), and 1 ml of the standardized cell suspensions was used to inoculate 20 ml LB. Cultures were incubated at 37°C, shaking at 150 rpm, and the OD_{600} was measured at 2 h intervals for 16 h.

4.2.5 Determination of Tn insertion sites

The location of the insertion site was determined by cloning and sequencing of the transposon insertion site (Fig. 5). Chromosomal DNA from mutant 5B9 was obtained using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions. The chromosomal DNA (0.4 µg) was digested with 20 U EcoRI (Fermentas) for 4 h at 37°C and purified using the QIAquick PCR Purification Kit (Qiagen). The fragments were then ligated into the plasmid pUC19. To prepare the plasmid DNA, EcoRI-digested pUC19 (0.57 µg) was dephosphorylated with 1 U of calf intestine alkaline phosphatase (CIAP) (Fermentas) at 37°C for 30 min and purified using

the QIAquick PCR Purification Kit. Fragments of mutant chromosomal DNA (0.14 µg) were ligated into the digested and purified pUC19 (0.05 µg) by incubating for 1 h at 22°C with 5 U of T4 DNA Ligase using the Rapid DNA Ligation and Transformation Kit (Fermentas). *E. coli* JM109 was then transformed using 5 µl of the ligation mixture (0.05 µg DNA) according to the manufacturer's instructions. Transformants containing the tetracycline resistance gene were selected on LB medium containing ampicillin (50 µg/ml), tetracycline (12 µg/ml), X-Gal and IPTG (Fermentas), and the yield of white colonies after transformation was 10^2 CFU/µg DNA. As a positive control for the ligation step, a portion of the transformed cells were plated on LB medium containing only ampicillin (50 µg/ml), X-Gal and IPTG, and the transformation efficiency was 10^3 CFU/µg DNA. A second control for the transformation process was prepared by transforming *E. coli* JM109 with 100 pg of supercoiled pUC19 DNA and plating on ampicillin (50 µg/ml), X-Gal and IPTG. The yield of blue colonies was $>10^6$ CFU/µg DNA.

Plasmids from the tetracycline resistant cells were isolated using the GeneJET Plasmid Miniprep Kit (Fermentas). To confirm the presence of the transposon, it was amplified using the PCR conditions and primers described above (section 4.2.2). The plasmid DNA (0.9 µg) was also digested with 20 U of *Eco*RI for 16 h at 37°C and analyzed by gel electrophoresis in order to estimate the size of the insert. Finally, the plasmid containing the insert was sequenced to determine the genes flanking the transposon.

4.2.6 Genomic sequence analysis

DNA sequencing was performed using automated sequencing (Mobix Lab, McMaster University, Hamilton, ON). Sequencing was carried out using Tn specific primers supplied with the EZ-Tn5 <Tet-1> Insertion Kit (Epicentre) (FP-1 5'-GGGTGCGCATGATCCTCTAGAGT-3'; RP-1 5'-TAAATTGCACTGAAATCTAGAAATA-3') and M13 Forward and Reverse universal primers. Sequences were analyzed using BLAST and compared to the *P. aeruginosa* PAO1 genomic sequence (Stover et al., 2000).

4.3 Results

4.3.1 Optimizing the growth of *L. monocytogenes* biofilms in microtiter plates

Incubation temperatures used to prepare inoculum for *Listeria* biofilms vary among different studies (Borucki et al., 2003; Lemon et al., 2007), and inoculum growth temperature has been found to affect the subsequent development of *L. monocytogenes* biofilms (Dykes, 2003; Francois et al., 2007). Therefore, to optimize biofilm growth two pre-incubation temperatures, 22 and 30°C, were examined. Although there was not a significant difference between the two temperatures, inoculum incubated at 30°C did result in a higher level of adhesion and was chosen for subsequent experiments (Fig. 6). The analysis of biofilm formation revealed a large degree of variation from one microtiter plate to another, and among the rows of biofilms within a single plate (Fig. 7).

4.3.2 Construction and screening of Tn mutants deficient in *Listeria* biofilm removal

To identify the genetic basis for removal of *L. monocytogenes* biofilms by *P. aeruginosa* PAO1, the supernatants of 1416 Tn mutants were screened, and six mutants that demonstrated a reduced ability to remove *Listeria* biofilms were selected for additional studies. First, the initial screening process using filter microtiter plates was repeated with eight replicates for each mutant. Results indicated that several mutants identified through the screening process had been false positives (data not shown). This same set of mutants was also used to prepare PCM as described in chapter three, by mixing sterile supernatant 1:1 with 2x concentrated TSB. Results confirmed that the majority of the mutants identified through the screening process had been false positives; however, one of the mutants was impaired in its ability to inhibit *Listeria* biofilms (mutant 5B9) (Fig. 8).

Mutant 5B9 removed significantly less biofilm than the wild-type PAO1 strain, and the amount of biofilm retained in the wells was not significantly different from the negative control. The growth curve for mutant 5B9 demonstrated a rate of growth that was comparable to that of the wild-type strain PAO1 (Fig. 9). To confirm the presence of the transposon, the tetracycline resistance gene was amplified by PCR (fig. 10).

4.3.3 Determination of transposon insertion site

The insertion site of the Tn mutation was determined by subcloning as attempts at inverse PCR were unsuccessful. EcoRI digested genomic DNA from mutant 5B9 was ligated into the cloning vector pUC19 and used to transform *E. coli* JM109. Colonies that were successfully transformed by plasmids containing the transposon insert were

identified by blue white screening and their growth on tetracycline plates. The relatively low transformation efficiency observed for transformants containing the Tn (10^2 CFU/μg DNA) is reflective of the small proportion of genomic DNA fragments harbouring the transposon.

To confirm the presence of the transposon, the plasmid DNA was recovered and the 1674 bp transposon was amplified by PCR. To determine the total size of the insert containing the transposon, the plasmid was digested with EcoRI and analyzed by gel electrophoresis. A band representing the 2686 bp pUC19 fragment was observed in addition to the insert, which was revealed to be approximately 10 kb (Fig. 11).

Sequencing results were compared to the *P. aeruginosa* PAO1 genomic sequence. The ~1075 bp sequence obtained from the M13 forward primer corresponded to a partial sequence for PA3716 and the complete sequence for the small adjacent gene, PA3717 (Fig. 12). The M13 reverse primer gave ~900 bp sequence corresponding to the Tet-1 transposon. Results from the Tet FP-1 primer included in the EZ-Tn5 insertion kit produced a ~1025 bp sequence corresponding to the 19 bp mosaic end sequence of the transposon followed by a 918 bp segment of PA3724 (*lasB*) and a small portion of the non-coding region upstream from PA3723. This indicated that the transposon was located in the *lasB* gene. *lasB* is 1497 bp and codes for the secreted metalloprotease elastase (Stover et al., 2000).

4.4 Discussion

Transposon mutagenesis has been used to identify genes essential to a broad range of functions, including biofilm formation, virulence, motility, and anaerobic growth (Filiatrault et al., 2006; Li et al., 2007; Nian et al., 2007; Pearson & Hansen,

2007). It has also been used as an effective method to identify substances secreted by *E. coli* capable of inhibiting biofilm formation (Valle et al., 2006). In this study, random mutagenesis was used to elucidate the genetic basis of the *Listeria* biofilm inhibition demonstrated by *P. aeruginosa* PAO1.

4.4.1 Screening Tn mutants

A library of 1416 mutants was produced by random mutagenesis and screened for an impaired ability to remove *Listeria* biofilms. Prior to screening, the growth of *Listeria* biofilms in 96-well polystyrene plates was assessed to determine the degree of variation in the growth of the biofilms and the temperature at which the inoculum would be prepared. Inoculum grown 30°C was used in the subsequent experiments because it produced more biofilm than inoculum grown at 22°C, although the difference was not significant.

Analysis of *Listeria* biofilm growth on polystyrene revealed a large degree of variation. Results showed that there may be a significant difference between separate microtiter plates and among the biofilms within a single plate. Despite this variation, even the lowest levels of biofilm formation observed in TSB was significantly higher than the amount observed for biofilms treated with PAO1 PCM. Based on this observation, it was determined that the variance among control biofilms should not interfere with the preliminary screening process, and all candidates identified through the initial screening were subjected to additional tests.

The preliminary screening process produced six false positive results (0.4% of the mutants tested).These may have been a result of mechanical errors such as problems during staining and washing steps. Another possibility is that there was an

insufficient volume of sterile *Pseudomonas* supernatant filtrate transferred to the *Listeria* biofilms, as only small volumes of supernatant were recovered and the amount varied from well to well. Other complications known to occur in screening mutant libraries include non-null mutations in which a transposon insertion fails to fully eliminate target gene function, cross-contamination of one mutant by another in an adjacent well resulting in mutants being missed during screening, and polar effects where an insertion may reduce expression of downstream genes in an operon (Salama & Manoil, 2006).

4.4.2 Elastase LasB may be involved in the removal of *Listeria* biofilms

Based on sequencing results, it was determined that the transposon had disrupted the *lasB* gene. The product of *lasB, e*lastase, is a secreted zinc metalloprotease and a known virulence factor of *P. aeruginosa*. Elastase is capable of degrading or inactivating a range of biological tissues and immunological agents, including elastin, collagen, and immunoglobulin G (Adonizio et al., 2008; Bever & Iglewski, 1988). The elastolytic activity of elastase is associated with invasiveness of *P. aeruginosa* PAO1, due in part to its ability to disrupt the tight junctions of epithelial cells (Cowell et al., 2003). Expression of *lasB* is regulated by both the Las and Rhl quorum-sensing systems (Nouwens et al., 2003; Pearson et al., 1997; Schuster & Greenberg, 2006). Nouwens et al. (2003) demonstrated that production of elastase was present, although significantly reduced, in both Δ*las* and Δ*rhl* mutants, and only mutants with deletions from both QS systems failed to produce elastase. *P. putida* does not produce elastase (Braun et al., 2000), which is consistent with earlier observations that *P. putida* does not remove *Listeria* biofilms.

While it does appear that elastase is involved with the removal of *Listeria* biofilms, it remains to be determined if elastase inhibits *Listeria* biofilms directly, or is indirectly involved.

As elastase has a broad substrate range, is secreted from the cell, and is a major constituent of PAO1 supernatant, accounting for approximately 60% of supernatant protein content (Nouwens et al., 2003), it is possible that elastase directly inhibits *Listeria* biofilms.

The temperature dependence of the biofilm removal reported in chapter three suggested that treatment with PAO1 conditioned medium may impair motility of *Listeria,* either by repressing the expression of flagella or by altering their function, as flagellar motility has been reported to be an important factor in *Listeria* biofilm development (Lemon et al., 2007). Interestingly, in *P. aeruginosa* neutrophil elastase (NE) has been reported to impair motility by suppressing synthesis of flagellin, the principle component of bacterial flagella. Transcription of flagellin in *P. aeruginosa* depends on prior assembly of the flagellar hook basal body, and NE destroys the flagellar hook, therein repressing the expression of flagellin at the transcriptional level (Jyot et al., 2007). NE was found to have no effect on *P. aeruginosa* viability, just as PAO1 conditioned medium had no effect on the viability of *Listeria* in this study (Sonawane et al., 2006). This suggests the possibility that LasB elastase may directly affect *Listeria* flagella by degrading components of the flagella itself, or by affecting other factors normally involved in the anti-repression of flagella observed at temperatures below 37°C, such as GmaR or DegU (Shen et al., 2006).

Elastase is known to have a broad range of substrates (Anderson et al., 1999), and even structures on the surface of *P. aeruginosa* have been found to be degraded in the presence of its own stationary phase supernatant. Quorum sensing mutants of *P. aeruginosa* unable to synthesize elastase were found to have significantly increased expression of membrane proteins, including flagellin and a flagellar hook associated protein, FlgK (Nouwens et al., 2003). Thus elastase may be acting directly to degrade components of *Listeria* cell surface (Nouwens et al., 2002).

Alternatively, elastase may not be affecting *Listeria* directly, as activity of elastase has been demonstrated to affect other secreted substances from *P. aeruginosa*. Cowell et al. (2003) demonstrated that elastase degrades, and may prevent the secretion of ExoS, a type-III secreted protein produced by *P. aeruginosa* that inhibits its uptake of by epithelial cells. The authors hypothesize that elastase could be partially active in the periplasm where it may activate or degrade other proteins, or affect components of secretion machinery. Thus, it is conceivable that elastase may be involved in activating other substances that are involved in the removal of *Listeria* biofilms.

Whether elastase is directly or indirectly responsible for the biofilm inhibition, it is possible that additional factors, unrelated to elastase, may be involved. Although treatment with Mutant 5B9 PCM did not result in a significant reduction in biofilm, the amount was lower than the negative control; therefore, other substances secreted by *P. aeruginosa* may also play a role in the complete removal of the biofilms. For example, Irie et al. (2005) found that rhamnolipids disrupted *Bordetella* biofilms, however, when experiments were conducted with purified rhamnolipids, as opposed to *Pseudomonas*

conditioned medium, the purified rhamnolipids were less effective at biofilm removal and it was suggested that the difference was likely due to other additional substances present in the conditioned medium. It may be that other substances in addition to elastase, perhaps rhamnolipids, are also involved in the removal of *Listeria* biofilms.

One possible candidate is LasA, a staphylolytic protease. Full expression of the elastolytic phenotype in *P. aeruginosa* is dependent on the expression of both elastase and LasA (Kessler et al., 1993). Together LasA and elastase function in a synergistic manner, however, LasA does exhibit a low level of elastolytic activity on its own (Cowell et al., 2003). Furthermore, it was reported that mutation of *lasB* reduced invasiveness of *P. aeruginosa* by 77%, whereas mutation of *lasA* resulted in a 70% reduction (Cowell et al., 2003). These findings indicate that LasA and elastase can produce similar effects, and the activity of LasA may contribute to reducing the amount of *Listeria* biofilm compared to the negative control.

An additional possibility is that the effect of some substances in the supernatant may become more prominent in the absence of elastase. Elastase is normally the principle component of *P. aeruginosa* supernatant, thus the conditioned medium from the Mutant 5B9 would differ considerably in its composition compared to the wild-type. The loss of LasB, and its proteolytic activity, results in greater amounts of secreted proteins with longer half-lives (Nouwens et al., 2003), some of which may be affecting the *Listeria* biofilms in ways that would not occur in the wild-type.

To narrow down these possibilities, additional tests carried out with purified elastase would be necessary to determine if it is directly involved in removing *Listeria* biofilms. In addition to investigating the effect of elastase, to gain a more complete

picture of the genes involved in removal of *Listeria* biofilms, screening should also be conducted on a considerably larger scale. In this study, 1416 mutants were screened, however, due to the large size of the *P. aeruginosa* genome (6.3 Mbp) this represents only a small fraction of non-essential genes. For example, Jacobs et al. (2003) developed a saturated library of *P. aeruginosa* PAO1 consisting of 30,100 sequenced-defined transposon mutants. Similarly, Filiatrault et al. (2006) screened 35 000 random *P. aeruginosa* PAO1 mutants to identify genes involved in virulence and anaerobic growth. If additional tests reveal that elastase is not the primary substance involved in removing the *Listeria* biofilms, additional screening may lead to the identification of other factors that play a role in biofilm inhibition. On the other hand, if elastase does efficiently remove *Listeria* it may have potential for use in the prevention or removal of *Listeria* biofilms in food processing environments.

4.5 References

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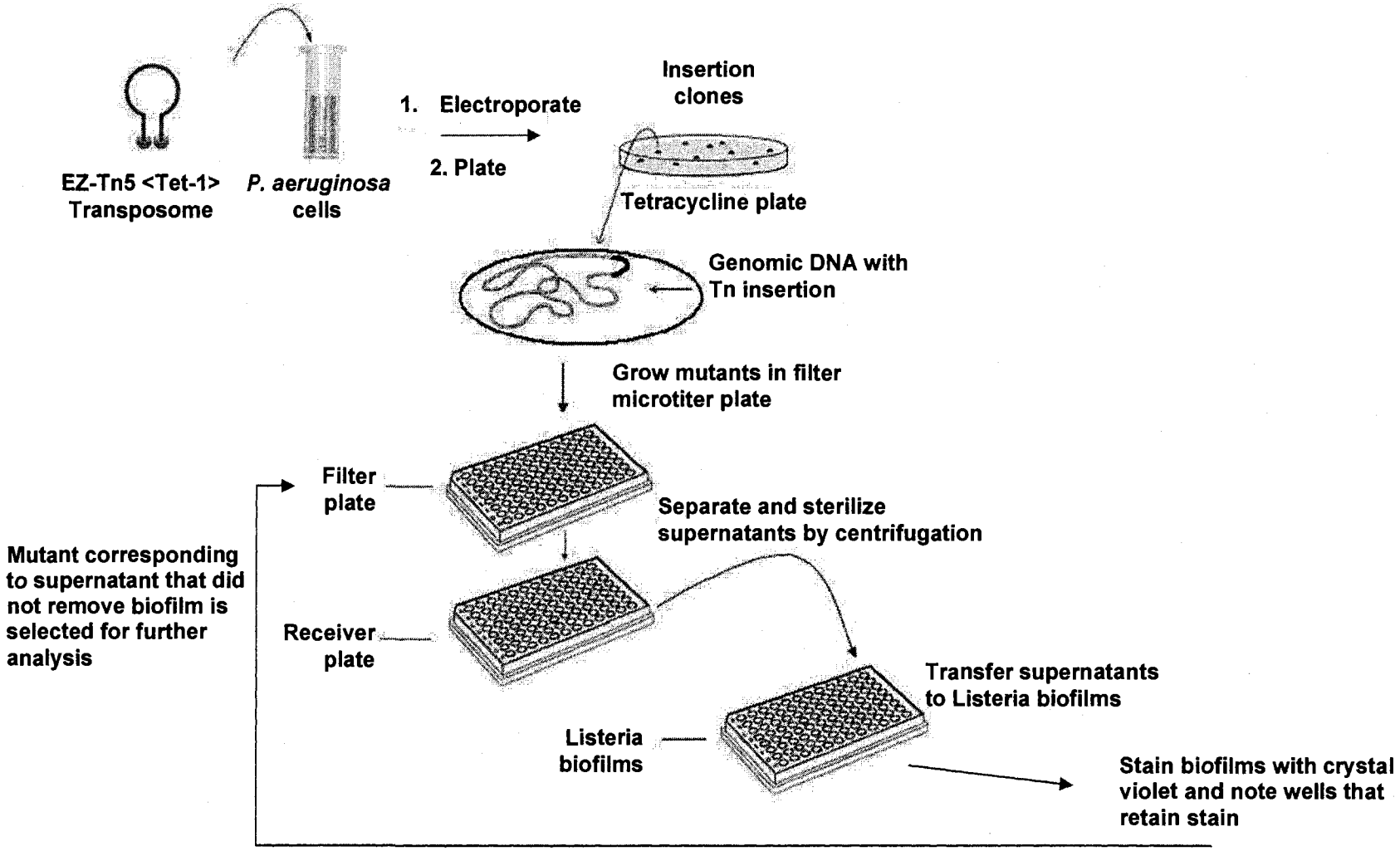

Figure 4. Generation and screening of random Tn5 mutants of *P. aeruginosa*

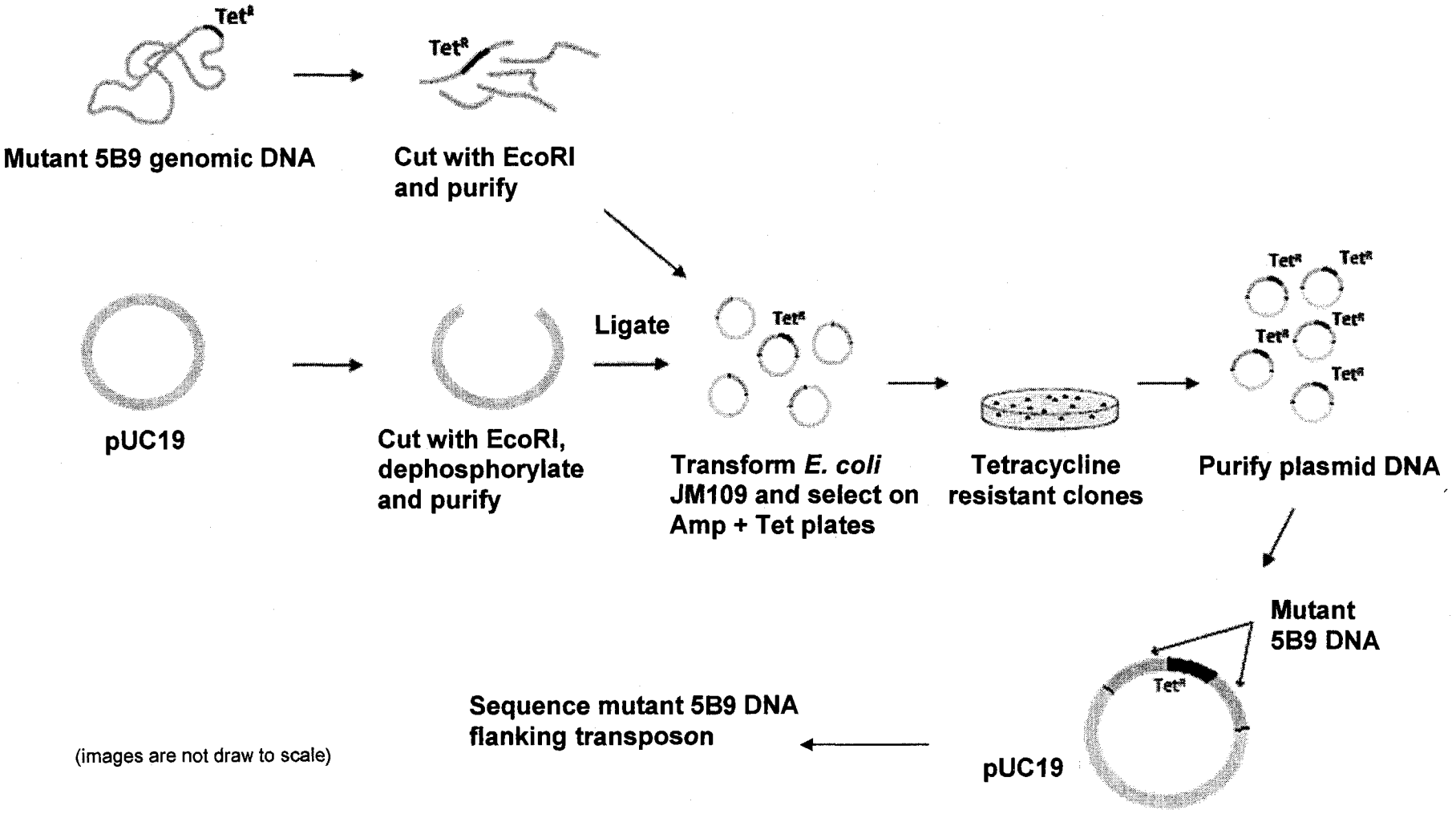

Figure 5. Procedure used to clone Mutant 5B9 genomic DNA into the cloning vector pUC19

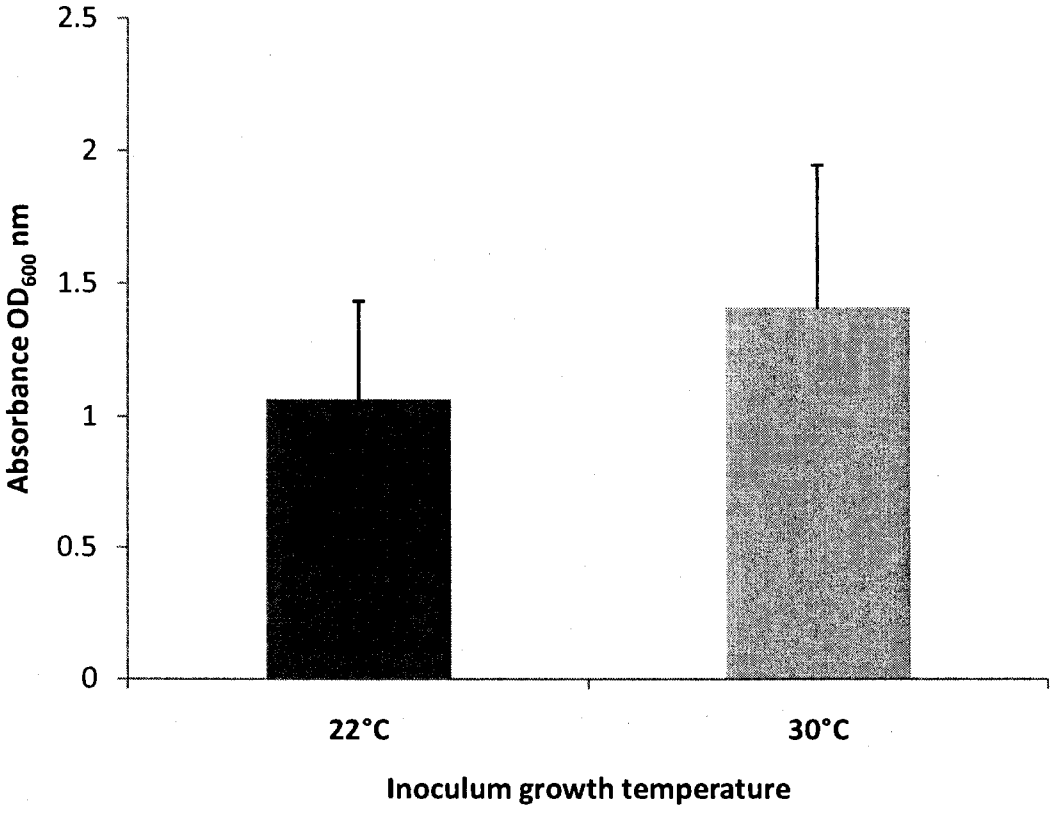

Figure 6. Biofilms formed on polystyrene using inoculum grown at either 22 or 30°C

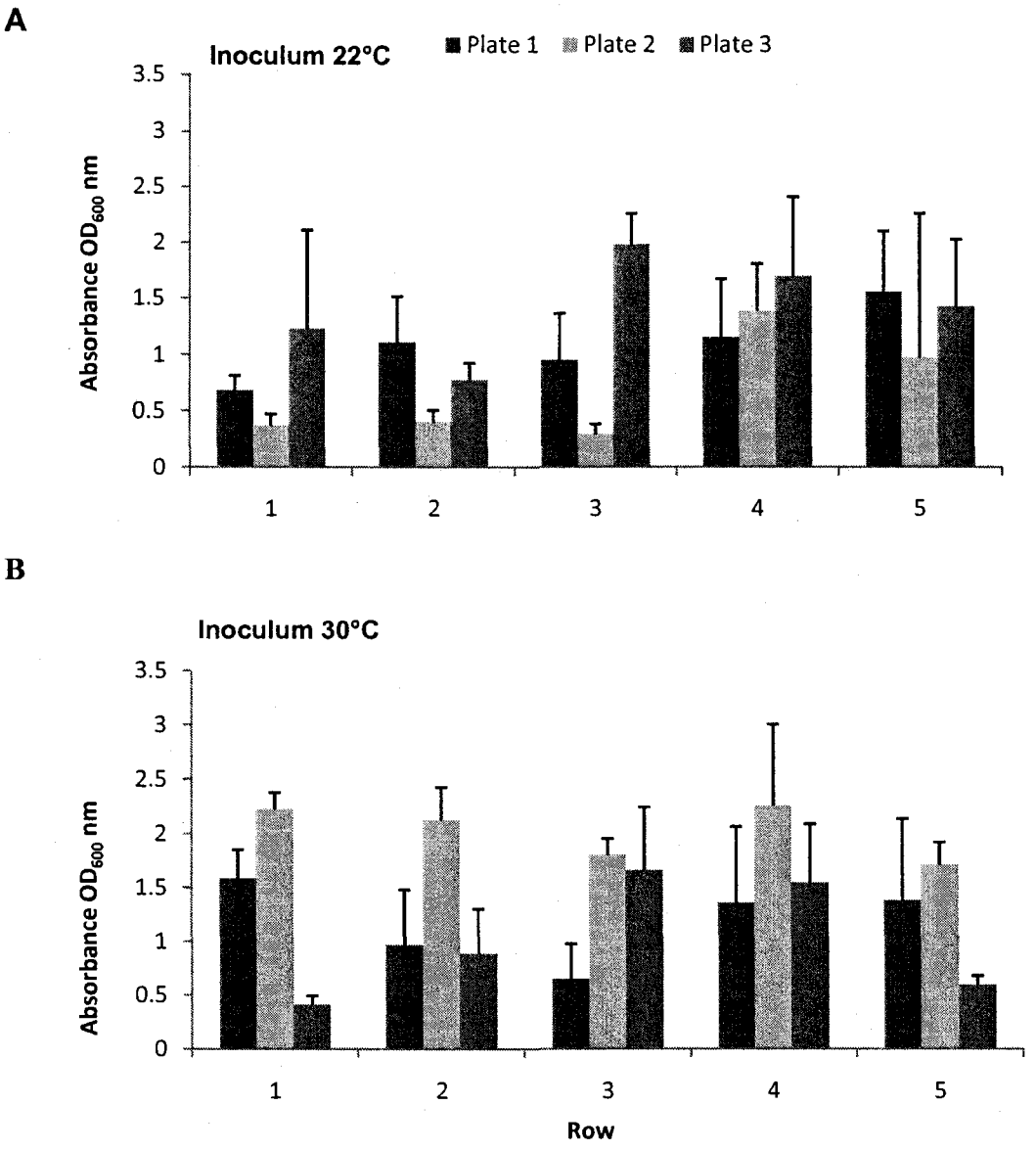

Figure 7. Variation in biofilm adhesion between individual rows within microtiter plates

Each point represents the average OD_{600} reading for 8 wells. Five rows of eight wells were tested for each plate. (A) Inoculum grown at 22°C, biofilm grown at 30°C. (B) Inoculum grown at 30°C, biofilm grown at 30°C.

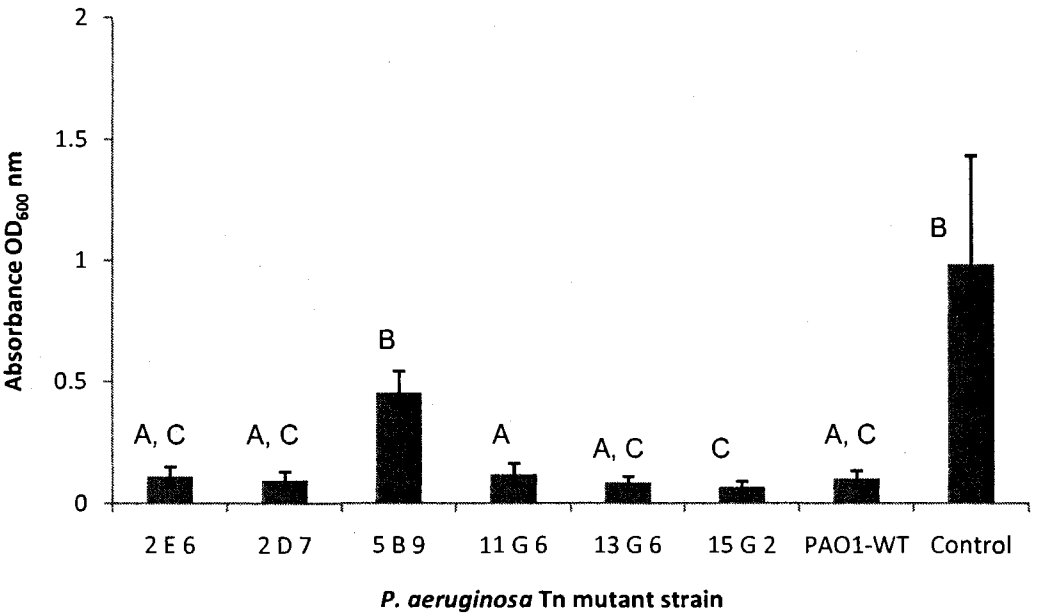

Figure 8. 24 h *Listeria* biofilms grown in 96-well plates exposed to mutant PCM

Each well represents the average OD_{600} reading for 16 wells. Different letters indicate significantly different values, ANOVA ($p < 0.05$).

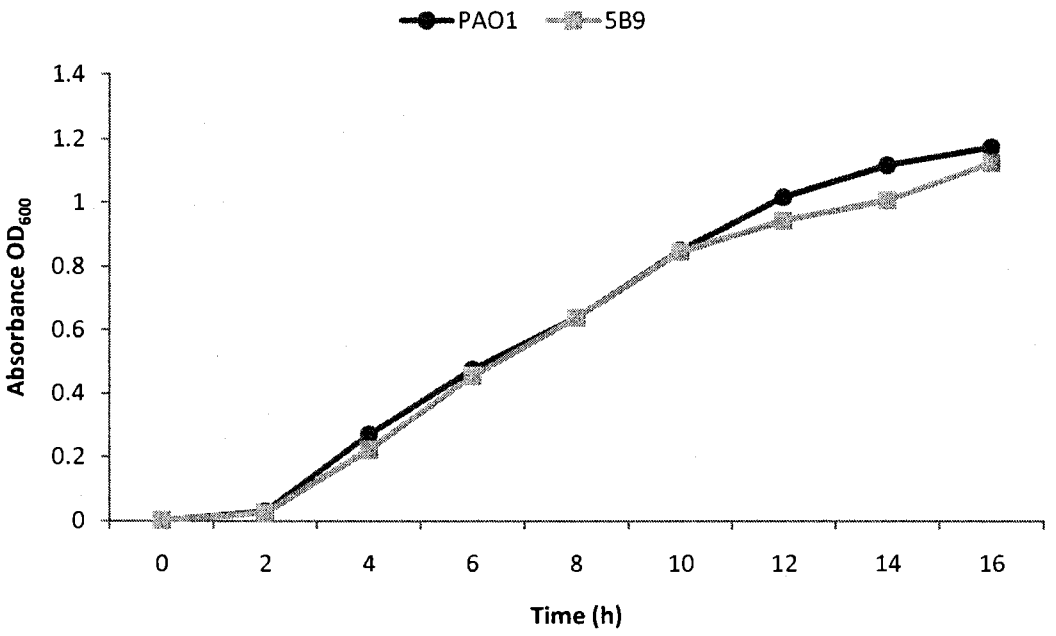

Figure 9. Growth curves of Mutant 5B9 and wild-type *P. aeruginosa* PAO1

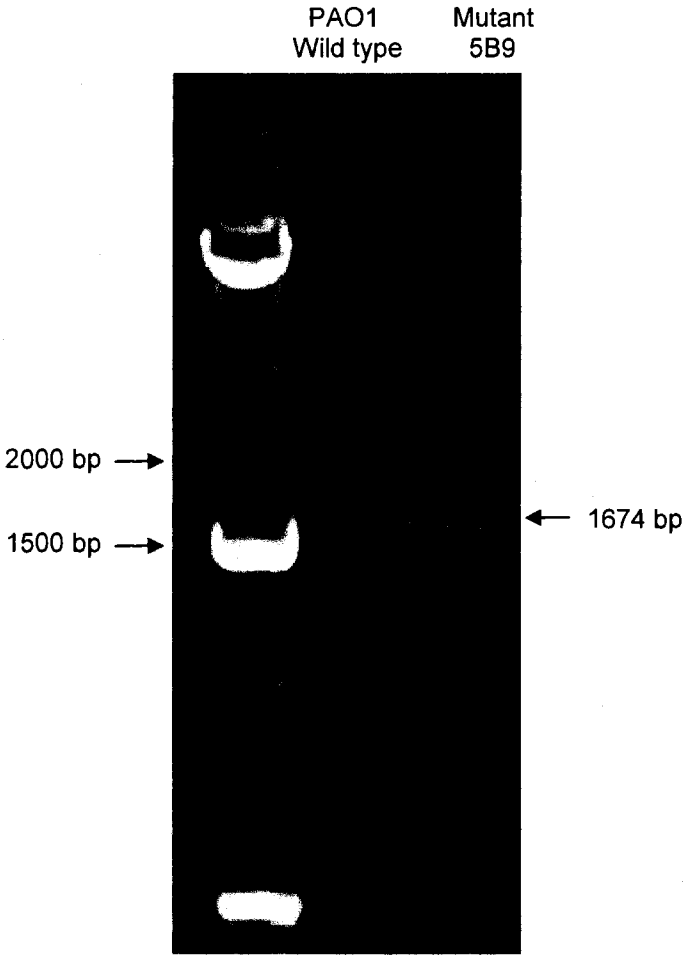

Figure 10. PCR amplification of the EZ-Tn5 <Tet-1> transposon

Lane 1: ladder; lane 2: PAO1 wild-type; lane 3: amplification of the tetracycline resistance gene of the Tn5 trsposon in mutant 5B9.

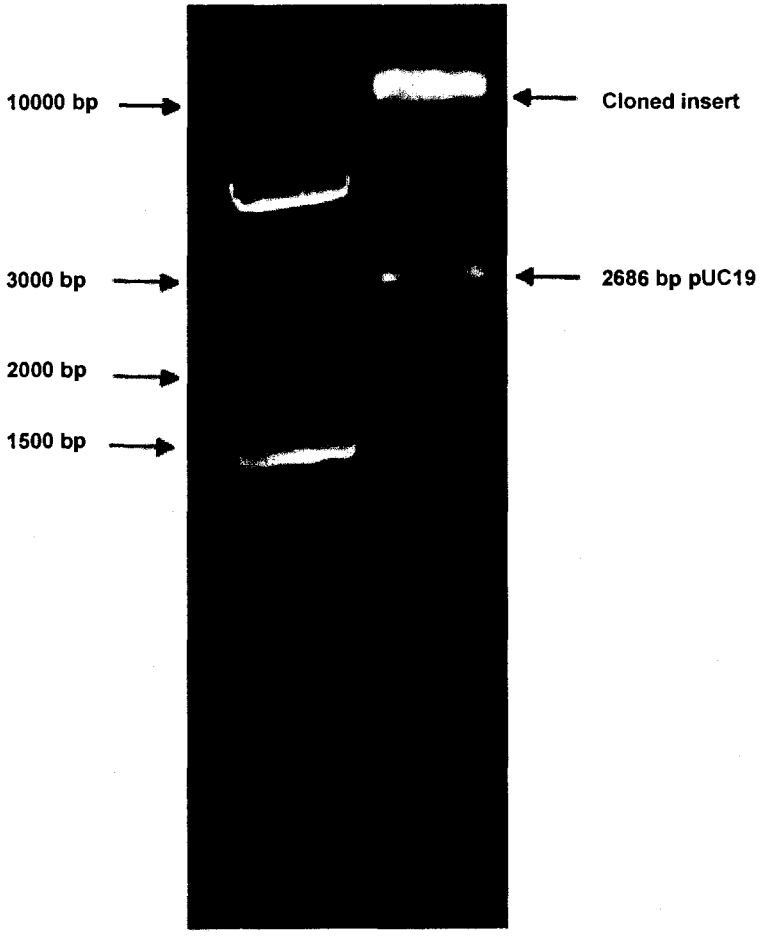

Figure 11. Insert cut from the pUC19 plasmid with EcoRI

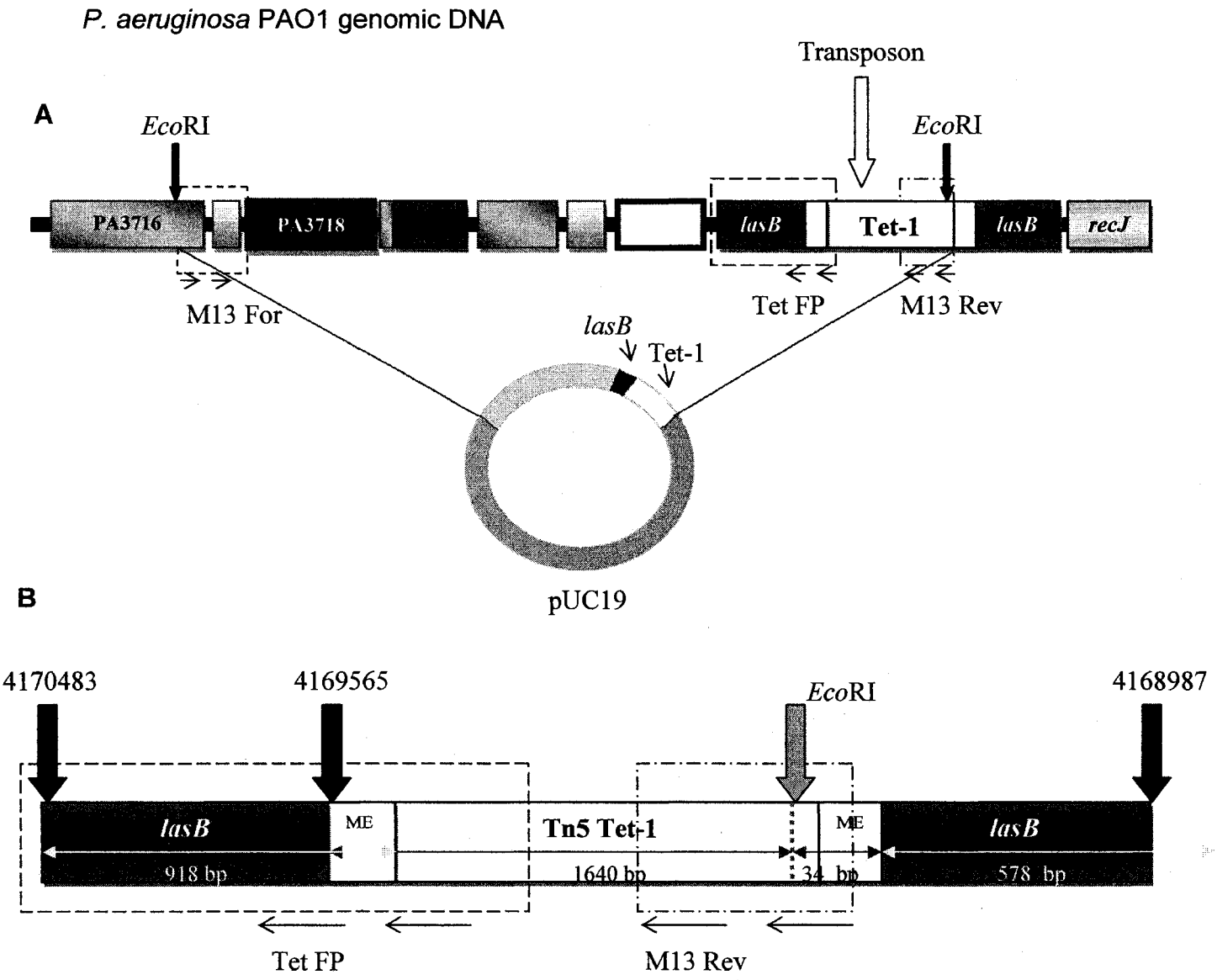

Figure 12. Location of the Tn5 Tet-1transposon

(A) Fragment of *P. aeruginosa* genomic DNA containing the Tn cloned into pUC19. (B) Location of the transposon in the *lasB* gene. Black arrows indicate the position of lasB and the transposon in the genome. The ends of the transposon are flanked by two 19 bp mosaic end sequences (ME) that can be used as a marker to determine the end point of the transposon (images are not drawn to scale).

5. Conclusions

The main findings of our study are that: i) substances secreted by *Pseudomonas* are capable of altering the growth of *Listeria* biofilms; ii) substances produced by *P. putida* enhance the attachment of *Listeria* biofilms grown at 37°C; iii) *P. aeruginosa* secretes substances that prevent and remove *Listeria* biofilms grown at temperatures ≤ 30°C, and the secreted virulence factor elastase plays a role in this removal.

At the onset of our study our goal was to identify a novel secreted substance capable of preventing or removing *Listeria* biofilms. A variety of microbially secreted substances produced by different species have been reported as having anti-biofilm effects, either preventing adhesion or dispersing mature biofilms. A common characteristic among many of these substances is that they exhibit surface active properties; therefore, in search of a novel substance to control *Listeria* biofilms, we screened bacteria isolated from paper mill slimes for biosurfactant production. While this type of broad screening may lead to the discovery of novel substances, and even new organisms, it is a large undertaking that can involve a lengthy investigation.

We then turned our investigation to the well characterized and fully sequenced *P. aeruginosa* strain PAO1. The benefits of working with a fully sequenced organism were apparent when *P. aeruginosa* was found to remove *Listeria* biofilms and we were able to investigate this finding using random mutagenesis. As sequencing results could be searched against the genome database, the use of a sequenced organism facilitated rapid determination of the genetic factor involved in the observed biofilm removal.

Similarly, the observation that *P. putida* secretes substances that enhance the attachment of *Listeria* could be further investigated using random mutagenesis, as was

done with *P. aeruginosa*. The use of a sequenced strain of *P. putida* would make this type of investigation possible, and could potentially identify the genetic basis behind the enhanced attachment of *Listeria*.

To gain a better understanding of the effect of *lasB* on *Listeria* biofilms, additional analysis will be necessary. A critical step to determining the role of elastase in biofilm removal will be to find out if it is directly or indirectly involved, by introducing elastase into a *Listeria* biofilm, apart from *Pseudomonas* conditioned medium. To do this, *lasB* could be cloned into another species and used to produce conditioned medium; however, elastate is secreted as a proenzyme, and attempts to express it in *P. putida* and *P. fluorescens* have failed to produce the functional enzyme. The mechanisms behind the dissociation of the propeptide from the enzyme are not well understood, but it is believed to be a factor supplied by *P. aeruginosa*'s secretion machinery that is not present in the heterologous hosts (Braun et al., 2000). Alternatively, another strategy would be to produce purified elastase from culture supernatants (Mariencheck et al., 2003).

A further area of interest will be to test the effect of the conditioned medium with a variety of Gram-positive and Gram-negative bacteria. Substances secreted by *P. aeruginosa* have been previously reported as having antimicrobial and anti-biofilm activity against a number of species (Nitschke & Costa, 2007) and, as this study demonstrates, it is likely effective against a number of organisms yet to be identified. It would be worthwhile to continue investigating the effects of *Pseudomonas* secreted substances, and elastase, on adhesion and biofilm formation and tests with other organisms problematic to the food industry would be of particular interest.

To determine if the motility is being affected as hypothesized, the mechanism behind the dispersal of *Listeria* biofilms could be investigated. One approach to this would be through microarray analysis, to examine how *Listeria* gene expression is affected by exposure to PAO1 PCM, and to elastase. Understanding how PAO1 PCM mediates dispersal of the biofilms may identify vulnerabilities in *Listeria* that could then be targeted with additional substances, in addition to giving broader insights into factors involved in biofilm formation.

Controlling the adhesion of microorganisms to food contact surfaces is essential to ensuring food safety, and due to the difficulties encountered in biofilm removal there is ongoing research into novel strategies for biofilm control. It has been suggested that the use of substances that are able to disperse biofilms in combination with antimicrobials may be an effective strategy to overcome the inherent resistance of biofilm cells (Irie et al., 2005; Nitschke & Costa, 2007). If it is established that elastase is directly responsible for the dispersal of *Listeria* biofilms observed in this study, it could potentially be employed in such an antimicrobial strategy.

In addition to identifying a substance that shows potential as a novel antilisterial treatment and contributing to the knowledge of how the growth of *Listeria* biofilms is influenced by other organisms, the findings of this study should serve as a solid foundation for future investigations regarding the interactions between *Pseudomonas* spp. and *Listeria monocytogenes*.

5.1 References

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