MODULATION OF *LISTERIA MONOCYTOGENES* BIOFILM FORMATION USING SMALL MOLECULES AND ENZYMES

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By

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ABSTRACT

Inadequately disinfected food contact surfaces colonized by *Listeria monocytogenes* can come into contact with ready-to-eat food products causing cross-contamination and food-borne outbreaks. *L. monocytogenes* is tolerant of high salt, low temperatures and low pH, in part due to its ability to form biofilms, defined as communities of microorganisms that are surrounded by a self-produced extracellular polymeric substance that can adhere to surfaces. Biofilm formation is a complex process involving a series of poorly defined physiological changes that together lead to tolerance of disinfectants and antibiotics. To better understand the process of *L. monocytogenes* biofilm development, and to investigate ways in which colonization of surfaces might be prevented, we developed a microtiter biofilm assay suitable for high throughput screening. The assay was used to identify small molecules (protein kinase inhibitors and previously FDA-approved bioactive drugs) that modulate *L. monocytogenes* biofilm development. Of the subset of molecules with biofilm modulatory activities, we showed that select protein kinase inhibitors and β-lactams prevented or reduced biofilm formation (<50% of vehicle control) of *L. monocytogenes* lab strains and food isolates at micromolar concentrations. In other cases, specific β-lactams stimulated biofilm formation (>200% of vehicle control) at sub-minimal inhibitory concentrations. Characterization of the penicillin-binding protein

targets of the β-lactams revealed that PBPD1, a low molecular weight D,D-carboxypeptidase, is targeted by non-stimulatory β-lactams. Stimulatory β-lactams did not increase biofilm formation of a *pbpD1* mutant to the same extent as wild type. In addition to inhibiting biofilm formation, many β-lactams dispersed established biofilms, although not completely. However, targeting components of the EPS matrix with enzymes — specifically proteins, using proteinase K –completely blocked adhesion and removed established biofilms. Together, these findings demonstrate how molecules with different mechanisms of action can modulate biofilm formation and the potential for use of proteinase K in the food industry as an antibiofilm agent.

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LIST OF ABBREVIATIONS

^{o}C	degree Celsius
%	percent
Δ	delta, deletion
>	greater than
<	less than
~	approximately
1D/2D	one-/two-dimension
Agr	accessory gene regulator
AI-2	autodinducer-2
ANOVA	analysis of variance
ATP	Adenosine triphosphate
AU	arbitrary units
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
β	beta
Bap	biofilm associated protein
BHI	brain-heart infusion medium
Boc-FL	Bocillin-FL
C-terminal	carboxyl-terminal
cfu	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
cm	centimetre
CO_2	carbon dioxide
CV	crystal violet
Da	daltons
d	day(s)
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSF	diffusible signalling factors
E. coli	*Escherichia coli*
eDNA	extracellular deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid

EPS	extracellular polymeric substance
Erm	erythromycin
ESI	electrospray ionization
FDA	Food and Drug Administration
GRAS	generally regarded as safe
h	hour(s)
H-factors	flagellar factors
HRMS	high-resolution mass spectrometry
HMW	high molecular weight
IC_{50}	half maximal inhibitory concentration
Inl	internalin
J	coupling constant
KCl	potassium chloride
kDa	kilodaltons
KH_2PO_4	monopotassium phosphate
kV	kilovolts
L. lactis	*Lactococcus lactis*
L. ivanovii	*Listeria ivanovii*
L. monocytogenes	*Listeria monocytogenes*
LB	Luria-Bertani medium
LC	Liquid chromatography
LMFI	*Listeria monocytogenes* food isolate
LMW	low molecular weight
LPXTG	leucine-proline-x-threonine-glycine
LTA(s)	lipoteichoic acid(s)
μg	microgram(s)
μL	microlitre(s)
μm	micron(s)
μM	micromolar
m-DAP	*meso*-diaminopimelic
m/s	metres per second
m/z	mass-to-charge ratio
mg	milligram(s)
MHB	Mueller Hinton Broth
MIC(s)	minimal inhibitory concentration(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)

mM	millimolar
MS	mass spectrometry
N-terminal	amino-terminal
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
ng	nanogram(s)
nm	nanometer
NMR	Nuclear magnetic resonance
NXXTX	asparagine –x-x-threonine-x
O-factors	somatic factors
OD	optical density
P. aeruginosa	*Pseudomonas aeruginosa*
P. fluorescens	*Pseudomonas fluorescens*
P. putida	*Pseudomonas putida*
PAD	previously-approved drugs
PBP(s)	penicillin-binding protein(s)
PBS	phosphate buffered saline
PG	peptidoglycan
pH	power of hydrogen
PKC	protein kinase C
PrfA	positive regulatory factor A
QS	quorum sensing
RNA	ribonucleic acid
RNA-seq	RNA-sequencing
rpm	revolutions per minute
RR	response regulator
RTE	ready-to-eat
s	second
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. pneumoniae	*Streptococcus pneumonia*
S. sanguis	*Streptococcus sanguis*
SCVs	small-colony variants
Sec	secretion
SEM	scanning electron microscopy
SK	sensor kinase

SNPs	single nucleotide polymorphisms
Srt	sortase
TCS(s)	two-component system(s)
TA	teichoic acid
TMS	tetramethylsaline
TSA	tryptic soy agar
TSB	tryptic soy broth
UV	ultraviolet
V	volts
VASP	vasodilator-stimulated phosphoprotein
vol	volume
wt	weight
WTA(s)	wall teichoic acid(s)
X	times
x g	times gravity

CHAPTER ONE

Introduction

Introduction

Food-borne pathogens infect millions of people each year, resulting in thousands of hospitalizations. In Canada, there were approximately 4 million cases of food borne illnesses per year in the last decade. Of these, 40% were caused by one of the 30 known pathogens and the remaining 60% by unspecified agents (1). *Listeria monocytogenes*, among the former group, has been involved in deadly outbreaks worldwide, including Canada (2). One of the largest Canadian food-borne outbreaks was caused by ready-to-eat (RTE) meat products contaminated by *L. monocytogenes* (3). Inadequately disinfected meat slicers resulted in the transfer of *L. monocytogenes* to RTE meat products. In total, there were at least 57 confirmed cases and 22 deaths (3). Since the 2008 outbreak, food products continue to be recalled due to *L. monocytogenes* contamination and outbreaks still occur worldwide (3, 4). Therefore, it is necessary to better understand the abilities of *L. monocytogenes* to adapt and survive on surfaces to prevent future outbreaks.

Listeria monocytogenes

L. monocytogenes is a Gram-positive, rod-shaped non-spore-forming facultative anaerobe (5, 6). It can survive temperatures between 1.0 to 50^{o}C, pH of 4.5 to 9.0, and high salt concentrations (10% NaCl) (7-9) and can grow as a saprophyte, intracellular pathogen or in biofilms (10-

12). *L. monocytogenes* is highly motile at 20-25^{o}C but at higher temperatures (37^{o}C), there is a decrease in flagellin production (13-15).

The genus *Listeria* contains 15 species, of which only two are pathogenic, *L. monocytogenes* and *L. ivanovii* (16-18). *L. monocytogenes* is clinically relevant to humans and *L. ivanovii* is associated with abortions in ruminants (17). *L. monocytogenes* is divided into four phylogenetic lineages (I, II, III, IV) based on ribotyping, virulence gene sequencing and multilocus enzyme electrophoresis (19-24), and 13 serotypes (23, 25). Lineage I contains *L. monocytogenes* serotypes 1/2b, 3b, 4b, 4d and 4e. Serotypes 1/2a, 1/2c, 3a and 3c are in lineage II. Lastly, lineage III and the less characterized lineage IV are comprised of serovars 4a, 4b, and 4c (23, 25, 26). The majority of human listeriosis cases are caused by serotypes 1/2a (lineage II), 1/2b and 4b (lineage I) (27). Serotype 4b is the most common serotype isolated in clinical cases (17, 19, 23). Strains of lineage III and IV are mainly responsible for animal listeriosis cases (19, 25). The serotypes are classified via their somatic (O) antigen and flagellar (H) antigen patterns as defined by Seeglier *et al.,* and Paterson (8, 27-29). Serotypes 1/2a and 1/2b, which have been implicated in human listeriosis cases, express O-factors I, II and sometimes III. They differ in H-factor expression, where 1/2a expresses H-factors A and B, while 1/2b expresses H-factors A, B and C. Serotype 4b expresses O-factors V, VI and sometimes III, and H-factors A, B and C. The remaining serotypes are

distinguished from one another by their O-factors (I-IX) and H-factors (A-D) (8, 27, 28).

Listeriosis

There are several routes by which *L. monocytogenes* infects its host – through direct contact with an infected individual, animal to human transmission, mother-to-fetus transmission through the placenta, and most commonly, consumption of contaminated food (5, 9). Humans are most often infected through consumption of food products that become contaminated after thermal/heat-processing via contact with a contaminated surface, since *L. monocytogenes* does not survive at high temperatures (30). It takes approximately 10^4–10^6 *L. monocytogenes* cells per gram of ingested product to infect an individual, but the amount can be much less for the immunocompromised (31). It takes ~20 hours after consumption of infected food for the clinical course of infection to begin and once inside the host, the median incubation time is about 3 weeks (32, 33). Symptoms may manifest as meningitis, encephalitis, or septicaemia in invasive listeriosis cases (infection in the elderly and immunocompromised population). Symptoms of non-invasive listeriosis include gastroenteritis, headaches, nausea, or flu-like symptoms (5, 9, 32).

The change from a saprophyte to an intracellular pathogen requires the activation of the positive regulatory factor A (PrfA). PrfA is located on a

'*Listeria* pathogenicity island' along with other virulence genes responsible for intracellular invasion (10, 34). There are six genes in the pathogenicity island – *prfA, plcA, hly, mpl, actA,* and *plcB.* PrfA activates expression of the other virulence genes in the pathogenicity island (33), which are involved in the escape of the bacterium intracellularly following ingestion (5). In addition, PrfA controls the expression of the internalins (*inl*), which are surface proteins that bind to host cells (5, 35). Interestingly, deletion of the surface proteins, ActA and InlA, has opposing biofilm phenotypes. Deletion of ActA resulted in 55% biofilm reduction (36), whereas a deletion or truncated form of InlA stimulated biofilm formation (36, 37).

Currently there are no vaccines available and very few effective antibiotics to treat *L. monocytogenes* infections (31). Infected individuals are typically given ampicillin, penicillin, or for people allergic to penicillin, trimethoprim–sulpha-methoxazole or erythromycin intravenously (32, 38-40). Other antibiotics such as linezolid, vancomycin, or rifampicin have also been prescribed (40, 41). There are some antibiotics that have been successful at eliminating *L. monocytogenes in vitro,* but are not successful in mouse models, possibly due to the fact they may not be able to cross the biological barriers (32).

Biofilm formation

Foodborne pathogens, including *L. monocytogenes*, can form biofilms on various surfaces: plants, humans and food-processing machines (42-44). Biofilms are communities of microorganisms adherent to a surface and/or one another and surrounded by a self-produced extracellular polymeric substance (EPS) matrix (45). The EPS matrix of *L. monocytogenes* is composed of extracellular DNA (eDNA) and proteins (37, 46, 47). Exopolysaccharides are not typically part of *L. monocytogenes* EPS, as it lacks genes coding for exopolysaccharide production (46, 48). *L. monocytogenes* biofilm formation protects the cells from ultraviolet (UV) light, desiccation, sanitizing chemicals, and antimicrobial agents. Resistance to these agents allows the potential transfer of *L. monocytogenes* from equipment to food products (30, 49, 50). Biofilm formation is divided into five stages: reversible attachment followed by irreversible planktonic cell attachment to a surface, microcolony formation, maturation of the biofilm and lastly, dispersal (51-53) (Figure 1.1). Bacteria can use surface appendages (pili and/or flagella) and surface adhesion molecules for initial adherence (54-56). There are contradictory data regarding the role of flagella in *L. monocytogenes* biofilm formation. Flagella are expressed at 20^{o}C, but there is a reduction in flagella expression at 37^{o}C (15). Flagella were proposed to be critical for adherence to surfaces (14), while others showed that aflagellate

Figure 1.1

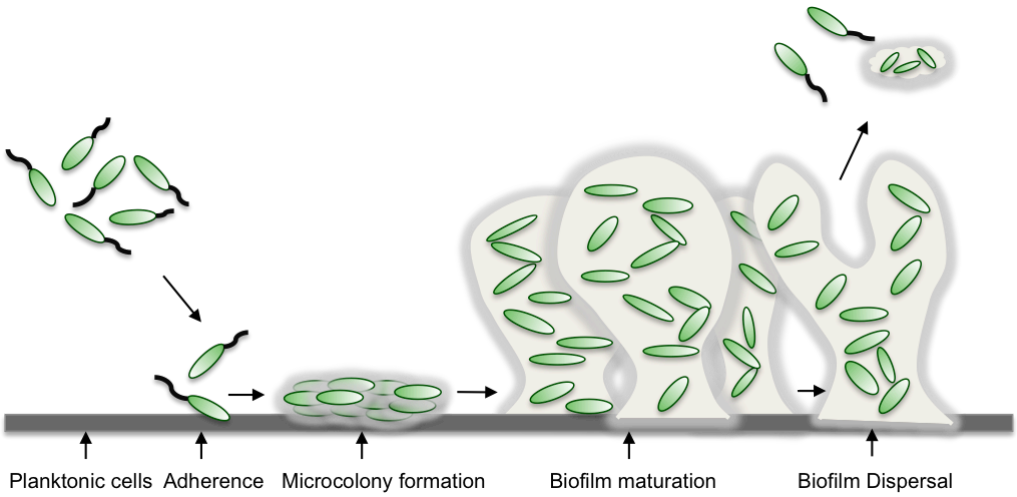

Figure 1.1. The stages of biofilm formation. There are 5 stages to biofilm formation (51-53): 1. Reversible cell adherence to a surface 2. Irreversible attachment of the cells along with the secretion of extracellular polymeric substances (EPS) 3. Development of small microcolonies 4. Mature biofilm development 5. Biofilm and single cell detachment. Figure adapted from O'Toole, G., et al. (Annu. Rev. Microbiol. 2000. 54:49–79).

mutants form (hyper)biofilms, depending on environmental conditions (57, 58). In addition, many studies have shown *Listeria* can form biofilms at 37^{o}C, a temperature where flagellum expression is repressed (36, 46, 58-60). In some strains of *L. monocytogenes*, a cell wall-anchored protein called biofilm-associated protein (Bap) is required for attachment (56). Bap homologues are found in both Gram-positive and Gram-negative bacteria and participate in biofilm formation and adherence to eukaryotic cells (61).

Interestingly, in *L. monocytogenes*, Bap is not an important adhesion factor for biofilm formation as *bap*-negative *L. monocytogenes* strains formed biofilms, and in some cases, adhered better than *bap*-positive strains (56).

Following irreversible attachment, quorum sensing (QS) – a process that allows bacterial cells to communicate with one another in a cell-density dependent manner using small signal molecules (autoinducers) – activates genes involved in adherence and EPS production (62, 63). There are two QS systems in *L. monocytogenes*: the peptide-mediated accessory gene regulator (Agr) system and the auto-inducer 2 (AI-2) LuxS system (64-67). In Gram-positive bacteria, a transmembrane protein or other protease processes the signal molecules, which are then secreted. Once secreted, autoinducers accumulate and when concentrations reach a critical threshold, interact with the sensor histidine kinase of a two-component regulatory system. Binding of the autoinducer results in the propagation of the signal to a cytoplasmic regulator through a phosphorylation cascade. The phosphorylated regulator then modulates the transcription of specific genes (65). There are four genes in the Agr system, *agrA, agrB, agrC,* and *agrD,* encoding a two-component histidine kinase (AgrC), a response regulator (AgrA), and a precursor peptide (AgrD) that is processed into the autoinducer by AgrB. In *L. monocytogenes*, deletion of *agrA* and *agrD* resulted in decreased

adherence and affected biofilm formation only within the first 24 h (67). In contrast, the Agr system in *Staphylococcus aureus* appears to block biofilm formation, as *agr*-negative strains showed enhanced biofilm formation (68). Although identified as a QS system, the autoinducer-2 (AI-2) LuxS system does not appear to play a quorum sensing specific role in *L. monocytogenes* biofilm formation (65, 69).

After the cells attach and form small microcolonies, they will develop into a mature biofilm. *L. monocytogenes* biofilm morphology can vary. Small microcolonies, a homogenous layer of cells, honeycomb structures, or ball-shaped microcolonies surrounded by a network of knitted chains have been observed (70-72).

Environmental conditions within the biofilm eventually become unfavourable due to waste accumulation and depletion of nutrients, causing the cells within the biofilm to disperse (62). In addition, shear forces, depletion of oxygen, production of EPS degradation enzymes and surfactants, and upregulation of motility can all lead to biofilm dispersal (62, 73, 74). Some bacteria, such as *Pseudomonas aeruginosa* and *Xanthomonas campestris,* produce diffusible signalling factors (DSF), *cis*-unsaturated fatty acids that disperse both self-biofilms and biofilms of other bacteria (75-77). Biofilm dispersal releases the cells back into planktonic phase as single cells or small aggregates, allowing bacteria to restart the biofilm cycle (44, 62, 78).

Biofilm prevention and removal

There are many recent recalls of *L. monocytogenes*-contaminated food products (2, 79-81), demonstrating that current sanitizing agents are not completely effective at removing *L. monocytogenes.* Both chemical and mechanical approaches have been used to prevent and/or remove established *L. monocytogenes* biofilms, but they do not completely inhibit the growth of the cells that are released. Addition of EPS-degrading enzymes (DNase and proteases), medicinal and culinary herb extracts, cationic peptides, and surfactants have all been shown to reduce attachment of *L. monocytogenes* to various surfaces (46, 47, 50, 82-84).

Addition of DNase reduced initial attachment of *L. monocytogenes* on glass and biofilm formation on polystyrene. In addition, DNase dispersed 3 d old pre-existing biofilm on glass under flow cell conditions (46). Longhi et al. demonstrated that serratiopeptidase (SPEP), an extracellular metalloprotease, completely inhibited biofilm formation of many *L. monocytogenes* strains at 37^{o}C. At lower temperatures (25^{o}C and 4^{o}C), serratiopeptidase decreased initial attachment of the cells (47). Addition of 0.01% trypsin, a serine protease, reduced attachment of *L. monocytogenes* to Buna-N rubber and stainless steel surfaces by 99.9% (83). Protease K, another serine protease, dispersed 84-97% of pre-existing *L. monocytogenes* biofilm depending on the strains (37). Although

EPS-degrading enzymes prevented and dispersed existing biofilm, they do not kill the cells.

Natural products including rosemary, thyme, Echinacea, or peppermint reduced *L. monocytogenes* attachment by at least 50% on polyvinyl chloride. However, they are not as effective at inhibiting the growth of preformed biofilm. Only 3 of 15 extracts (rosemary, peppermint and tea tree) inhibited growth of established biofilms by at least 50%, and another 3 extracts (thyme, aloe, and cranberry) stimulated biofilm formation (50).

Cationic peptides and surfactants prevent attachment and biofilm development (59, 82, 84). We showed that a 9-amino acid cationic peptide called 1037 inhibited *L. monocytogenes* biofilm formation in a dose-dependent manner without killing the cells (84). Surfactants such as rhamnolipid, surfactin, N-lauroylsarcosine, and Triton X-100 reduce adhesion and biofilm formation (59, 82), but only purified rhamnolipid completely inhibited the planktonic growth of *L. monocytogenes*, suggesting a use for it to control *L. monocytogenes* (82).

One mechanical mean of removing *L. monocytogenes* biofilm is using ultrasound treatment. A 15 min ultrasound treatment at room temperature resulted in an 86% decrease in the levels of biofilm biomass on polystyrene and a 91% decrease in viable cells. When combined with

benzalkonium chloride, there was a further reduction in biomass levels and viable cells (85).

Identification of genes involved in *L. monocytogenes* biofilm formation

To prevent and remove existing biofilms, it is necessary to understand the mechanisms underlying *L. monocytogenes* biofilm development. Using *mariner* transposon mutagenesis, a genome-wide study was conducted to identify genes required for *L. monocytogenes* 10403S biofilm development. Mutations in genes involved in cell wall biosynthesis and homeostasis, flagella biosynthesis and motility, energy generation and metabolism, and transcriptional regulation significantly reduced biofilm formation (48). In addition, deletion of some of the genes under PrfA control (*hly*, and *actA*) as well as *prfA* itself impaired biofilm formation (36, 86). Other studies have inactivated genes in other cellular pathways to evaluate their role in biofilm formation (87-89). Knockout of *hrcA* and *dnaK*, which encodes the class I heat-shock response regulator and chaperone protein, respectively, reduced biofilm formation under continuous flow cell conditions (87). Deletion of *gltB* and *gltC* – involved in oxidative stress tolerance – reduced attachment and biofilm formation on plastic (89). Chang et al. (88) disrupted *lmo1386*, which encodes for a putative DNA translocase, to reduce biofilm by 45% under static microtiter

conditions. Thus, *L. monocytogenes* biofilm formation requires the regulation and coordination of many different genes and pathways.

Mechanisms of biofilm resistance

Compared to their planktonic counterparts, cells in a biofilm are up to 1000-fold more tolerant of antimicrobial agents for a variety of reasons, including reduced penetration of antimicrobials, decreased growth rate, activation of biofilm-specific genes and the presence of persister cells (90-93).

EPS matrix limits diffusion of antimicrobial compounds

The EPS is a barrier for many antimicrobial agents, restricting diffusion of the molecules. Enzymes secreted by the cells can degrade the trapped molecules, rendering them inactive; an example is degradation of β-lactams by β-lactamases (94). The decrease in diffusion reduces the effective antimicrobial concentration required to kill the biofilm cells that are located deep within the biofilm (45, 92, 95, 96).

Subinhibitory concentrations of antibiotics can act as signalling molecules to modulate gene transcription (97-100). One of the responses of bacterial cells to low levels of antibiotics is stimulation of biofilm formation, potentially as a protective response (101). Subinhibitory concentrations of tobramycin, tetracycline, and ciprofloxacin altered gene

expression in *P. aeruginosa* and induced biofilm formation (99). In other bacteria such as *Escherichia coli*, *Staphylococcus aureus* and *S. epidermidis*, exposure to antibiotics (e.g. β-lactams, aminoglycosides) increased production of EPS components (102-106), thus potentially leading to an increase in resistance.

Decreased metabolic activity to prevent killing by antimicrobial agents

The metabolic activity of cells located deep within the biofilm can be reduced because of the decrease in nutrient availability, thus leading to cells that are slow growing or dormant, a phenotype commonly associated with biofilm cells (95, 96, 107). Another factor that potentially contributes to slow growth in a biofilm is the activation of the general stress response, which allows the bacteria to survive environmental stresses such as heat, changes in pH, or osmolarity (95, 108). These slow growing or dormant cells are not killed by antimicrobial agents that target processes used only actively growing cells (92).

Increased resistance through activation of biofilm-specific genes

Many studies have demonstrated differences in the transcriptome of planktonic versus biofilm cells (109-111). It was hypothesized that the changes in gene expression during biofilm growth could aid in resistance (92). Using a whole-genome microarray, it was demonstrated there was at

least a twofold change in the expression level of 627 genes in *L. monocytogene*s EGD-e biofilms cells compared to the planktonic cells (112). Of the 627 genes, 342 genes were down-regulated (112). The down-regulated genes included those that are involved in nucleic acid and lipid metabolism and cell wall synthesis and division, suggesting that biofilms cells are in an inactive cellular state and can avoid killing by antimicrobial agents (112).

The 285 genes up-regulated in biofilm cells included enzymes involved in DNA recombination and repair; for example, excinuclease ABC and RecA (lmo1398) (112). In a continuous-flow *L. monocytogenes* biofilm, induced generation of genetic variants depended on RecA, which is involved in DNA repair and SOS stress response activation (113). In addition, *hrcA* and *dnaK* were up-regulated (112). As previously mentioned, HrcA and DnaK are class I heat-shock response regulator and chaperone proteins, respectively, and under static and continuous-flow conditions, they are required for wild-type levels of biofilm formation (87). It was suggested that *L. monocytogenes* biofilm formation required the activation of the stress response genes, which can lead to resistance against disinfectants (112).

Tolerance to antimicrobial agents via dormancy

The presence of ‘persister’ cells and small-colony variants makes it

difficult to eradicate biofilms (93, 114-116). Persister cells make up a small proportion of the biofilm population. Persisters are dormant and tolerant to the killing effects of antibiotics (116, 117), allowing for repopulation of the biofilm after antibiotic levels decrease. Their outgrowth gives rise to a population that is composed of both new persisters and cells that are as sensitive to the drugs as the originals, which indicates that these cells are not resistant mutants (116, 117). Small-colony variants (SCVs) are similar to persisters because they grow slowly and can easily be overlooked during environmental and clinical laboratory testing (114). In S*. aureus*, SCVs are one-tenth the size of normal colonies, do not produce coagulase, are deficient in electron transport and thymidine biosynthesis and are resistant to aminoglycosides and cell-wall active compounds, making it difficult to detect them and prevent recurrence of an infection (114, 118). In *L. monocytogenes*, small-colony variants have been observed (119, 120). Similar to *S. aureus* SCVs, *L. monocytogenes* SCVs are resistant to aminoglycosides. In addition, they are induced by sublethal concentrations of triclosan, sensitive to hydrogen peroxide, and have decreased hemolytic activity (119, 120).

The cell surface of *Listeria*

For biofilm formation to occur, cells must adhere to a surface (51-53). Attachment requires the expression of adhesive components that can

bind to chemically diverse surfaces. These components can be attached to the cell wall, and many have been implicated in attachment of *L. monocytogenes* to various surfaces (36, 56).

The Listeria peptidoglycan layer

L. monocytogenes is Gram-positive, thus it has a cytoplasmic membrane and peptidoglycan (PG) layer. Surface proteins and teichoic acids (TAs) can be attached to the membrane or PG (121). The isolated dry cell wall material of *Listeria* contains 30-40% PG (122), which is composed of alternating disaccharide sugar repeats of *N*-acetylmuramic acid (NAM)–(β-1,4)-*N*-acetylglucosamine (NAG). (121). The NAM residue has a stem pentapeptide (L-alanyl-γ-D- glutamyl-*meso*-diaminopimelyl-D-alanine-D-alanine) that can crosslink to another stem peptide (121, 123, 124). Interestingly, *Listeria* PG is similar to that of Gram-negative bacteria for two reasons – the presence of *meso*-diaminopimelic (*m*-DAP) acid in the third position of the stem peptide, and direct crosslinking of the stem peptides (Figure 1.2). In other Gram-positive bacteria, the third residue is L-lysine and the stem peptides are linked via a peptide bridge (121, 125).

Figure 1.2

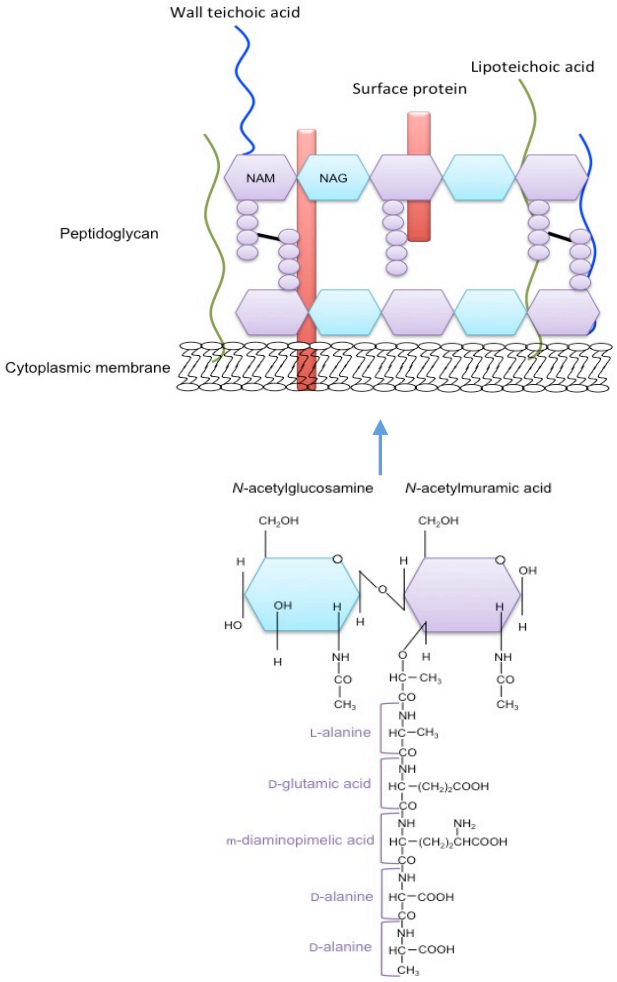

Figure 1.2. The cell surface of *Listeria*. The cell envelope of *Listeria* is composed of the cytoplasmic membrane and PG layer (repeating subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid linked by a β-(1,4)-glycosidic linkage). The stem peptide is linked to the lactyl group of NAM and can be directly cross-linked to another stem peptide. LTAs are anchored to membrane and WTAs are attached to NAM via a phosphodiester bond. Surface proteins can be covalently attached to NAM, non-covalently attached, or anchored to the membrane (121, 123, 124).

The effects of β-lactams on PBP function

PBPs are classified as high-molecular weight (HMW) or low-molecular-weight (LMW) (126). HMW PBPs are divided into class A PBPs, which have both transglycosylase and transpeptidase domains, and class B PBPs, which have a transpeptidase domain and a domain of unknown function that may have a role in interacting with other proteins during cell morphogenesis (121, 126, 127). PG biosynthesis occurs in the cytoplasm with the synthesis of undecaprenyl-pyrophosphoryl-NAM-penta-stem peptide (lipid I). Lipid I is converted to lipid II by the addition of NAG from uridine diphosphate-NAG forming undecaprenyl-pyrophosphoryl-NAM-stem pentapeptide-NAG, which is then flipped to the outer leaflet of the cytoplasmic membrane via MurJ flippase (128). However, MurJ is absent in the *Listeria* genome (128).

The transglycosylase domain is involved in glycan chain formation, linking lipid II to existing PG, forming the β-1,4-glycosidic linkage between the sugars. The transpeptidase domain is involved in stem peptide crosslinking (121, 123, 129, 130). To control the extent cross-linking, LMW PBPs have D,D-carboxypeptidase and/or L,D/D,D-endopeptidase activity. They are involved in removing the terminal D-alanine from the stem peptide, and cleaving the cross-linked peptides, respectively (121, 127, 130-132). β-lactams, such as ampicillin, inhibit transpeptidation and carboxypeptidation because they structurally mimic the D-alanine-D-

alanine moiety of the stem peptide and can bind covalently to the active site serine of the PBPs. Due to the slow hydrolysis of the β-lactam ring, the PBPs are prevented from crosslinking stem peptides, which can lead to growth inhibition and eventually, cell death (124, 131, 133).

Bacteria have evolved various means of β-lactam resistance, including production of β-lactamases and PBPs with low affinity for β-lactams (126, 131, 134). β-lactamases, which are related to LMW PBPs, can inactivate β-lactams via rapid hydrolysis of the β-lactam ring (135, 136). In addition, structural rearrangement around the active site serine of PBPs can result in a lower affinity for β-lactams, but still allow for cross-linking of the stem peptide to continue (134, 137).

Identification of L. monocytogenes PBPs

Only 5 PBPs (PBP1-5) were described initially in *L. monocytogenes,* based on analysis of membrane samples labelled with radioactive penicillin (138-140). A subsequent study suggested that the genome of *L. monocytogenes* encodes 10 putative PBPs – two HMW class A (PBPA1 and A2), three HMW class B (PBPB1, B2 and B3), three LMW PBPs with D,D-carboxypeptidase activity (PBPD1, D2 and D3) and 2 β-lactamases (PBPC1 and C2). Using whole cell lysates and a fluorescently tagged penicillin, eight putative PBPs were identified. A 9th PBP (PBPD2) had activity only when overexpressed. No activity was

observed for PBPD3, thus it was not considered a PBP (141). To account for the discovery of new PBPs, the original PBPs 1-5 were renamed (Table 1.1) (141).

Table 1.1. The predicted PBPs of *L. monocytogenes*.

PBP[a]	Previous nomenclature	Gene	Molecular Weight (kDa)	Predicted Function(s)
PBPA1	PBP1	*lmo1892*	90.87	Transglycosylase/ Transpeptidase
PBPB2	PBP2	*lmo2039*	81.89	Transpeptidase
PBPB1	PBP3	*lmo1438*	79.91	Transpeptidase
PBPA2	PBP4	*lmo2229*	77.85	Transglycosylase/ Transpeptidase
PBPB3	-----	*lmo0441*	74.60	Transpeptidase
PBPD1	PBP5	*lmo2754*	48.08	D,D-carboxypeptidase
PBPC1	-----	*lmo0540*	44.53	β-lactamase
PBPC2	-----	*lmo1916*	37.84	β-lactamase
PBPD3	-----	*lmo1855*	31.08	D,D-carboxypeptidase
PBPD2	-----	*lmo2812*	29.48	D,D-carboxypeptidase

[a] New nomenclature according to Korsak et al. (141)

The potential target of penicillin derivatives was hypothesized to be PBPB1 (formerly PBP3) due to their high affinity for this protein; it is the only PBP that is identical in all *Listeria* species (138, 140). However, a recent study suggested that PBPB1 is not the primary target, because susceptibility of *L. monocytogenes* to β-lactams is not altered by either its

loss or overexpression (142). It was hypothesized that PBPB1 has a role in cell division, as its overexpression resulted in shorter cells in stationary phase (142). PBPB1 is orthologous to PBP2A of *Bacillus sp.*, which is involved in formation of rod-shaped cells from oval spores. Mutagenesis of other PBPs (PBPA1, A2, B3 and D1) alters *L. monocytogenes* morphology causing cell chaining, increased cell length and cell wall thickness, and/or curving of the cells (143, 144).

The roles of autolysins in peptidoglycan breakdown and biofilm formation

Autolysins are involved in the breakdown of PG during growth, cell division and lysis. They cleave the β-1,4-glycosidic bond between the sugars, the bond between the glycan chain and stem peptide, and bonds between the peptides (121). There are three *Listeria* proteins with putative *N*-acetylglucosaminidase activity, involved in cleaving the bond between NAG and NAM. Six more proteins have putative *N*-acetylmuramidase activity, hydrolyzing the bond between NAM and NAG. The bond between NAM and the stem peptide is hydrolyzed by *N*-acetylmuramyl-L-alanine amidases, four of which are encoded in the *Listeria* genome (121). Autolytic activity is important for biofilm formation in bacteria such as *S. aureus* and *Lactococcus lactis*. An increase in *S. aureus* biofilm formation in the presence of sub-MIC methicillin requires autolysins to release

eDNA, while in *L. lactis*, a mutant deficient in cell wall hydrolase activity was unable to form biofilms (104, 145).

Surface molecules: Incorporation into the cell wall and role in biofilm formation

Surface proteins and TAs can be attached to PG (Figure 1.2). The genome of *L. monocytogenes* encodes ~60 putative surface proteins (47, 121, 146, 147). The export of proteins across the cytoplasmic membrane can be completed by seven systems with the secretion (Sec) system considered the main protein export pathway because over 700 proteins are predicted to have an N-terminal signal peptide (148-151). The surface-bound proteins can be classified as covalently anchored to the PG by sortases; non-covalently attached; and membrane anchored (121). Covalently anchored LPXTG-motif surface proteins (over 40), including InlA, are the best-studied class. Sortase substrates have an N-terminal signal peptide mediating transport via the Sec pathway and a C-terminal sorting signal consisting of the LPXTG motif, a hydrophobic domain, and a positively charged tail (121). Sortase (SrtA), a membrane bound transpeptidase with an active site cysteine, recognizes the LPXTG motif, cleaving between threonine and glycine and catalyzing the formation of an amide bond between threonine and *m*-DAP of the stem peptide. The surface protein, once linked to cell wall precursors, is incorporated into the

PG (121, 130, 152). In addition, there is a second sortase (SrtB) that recognizes a NXXTX sorting signal. It has two substrates, Lmo2185 and Lmo2186 (153). Non-covalent proteins bind to cell wall through repeat domains and include InlB and cell wall hydrolases. Lastly, membrane bound proteins such as ActA are anchored by hydrophobic tails or in the case of lipoproteins, anchored via covalent N-terminal lipididation (121, 154). ActA has been demonstrated to be required for *L. monocytogenes* biofilm formation and colonization in the gut lumen of mouse models (36, 155)

TAs, divided into wall teichoic acids (WTAs) and lipoteichoic acids (LTAs), are polyanionic polymers that make up a large proportion of cell wall carbohydrates in Gram positive bacteria (156). *L. monocytogenes* WTAs are polymers of ribitol-phosphate that are linked to NAM via a phosphodiester bond. Depending on the serotype, there can be modifications on ribitol (NAG, rhamnose, D-alanine, glucose, and/or galactose) (122, 156). LTAs are amphipathic as they have both hydrophilic and hydrophobic regions. The hydrophilic section is composed of 1-3 phosphodiester-linked glycerol-phosphate polymers substituted with galactose and D-alanine. The hydrophobic region is composed of glycolipids (galactose bound to glycerol and substituted with fatty acids), anchoring LTAs to the head group of membrane lipids (121, 122). One of the substituents in both TAs, D-alanine is important for cation homeostasis

and resistance to cationic antimicrobial peptides because it decreases the overall negative charge on the cell surface (123, 157). Disruption of WTAs have been shown to increase autolytic activity in *S. aureus* (158). It was hypothesized that WTAs indirectly target the autolysins to new PG, where WTAs are not present, for cell separation by blocking access to old PG, which have WTAs (158). In addition, the presence of WTAs intermediates at the septum appeared to initiate PBPs activities (159). In contrast, LTAs disruption was associated with a decrease in autolysin levels (160). In addition, mutations in the TA biosynthesis pathways reduced biofilm formation in many bacteria (48, 161, 162). Using a genome-wide screen approach, Ouyang et al. (48) reported that mutations in TA synthesis and modification reduced *L. monocytogenes* biofilm by 72-85%.

Two-component systems of *Listeria*

Under harmful environmental conditions, bacteria can respond by making biofilm to protect themselves from these stresses (73, 96). Bacteria respond and adapt to changing environment conditions by regulating gene expression. Other responses include changes to virulence, production of peptides or enzymes (163). The response to stimuli (ex. pH, antibiotics, temperature) is often mediated through two-component systems (TCSs). A typical TCS is composed of a membrane-anchored sensor histidine kinase and a cognate cytoplasmic response

regulator (164, 165). A sensor kinase has three domains: sensing, histidine kinase and ATP-binding (166). The sequence of the N-terminal sensing domain is variable because of the various signals the bacteria can perceive. After sensing the signal, the histidine residue in the highly conserved phosphotransfer domain is autophosphorylated in an ATP-dependent manner (166, 167). The phosphate is then transferred to the cognate response regulator, which has a receiver (regulatory) domain and an output (effector) domain. The regulatory domain has a conserved aspartic acid residue that receives the phosphate from the histidine residue of the sensor kinase, resulting in a conformation change in the output domain (167). The output domain is typically a transcription activator of a subset of genes (167).

The response regulators can possibly be phosphorylated by other histidine kinases because of the conserved nature of the receptor domain. The rate of phosphotransfer is reduced compared to cognate pairs (166). In addition, small molecule phosphodonors (ex. acetyl phosphate) can phosphorylate the response regulators (166, 168).

The genome of *L. monocytogenes* encodes for 15 sensor kinases and 16 response regulators. One of the response regulators, DegU, is considered an orphan response regulator as it is not associated with a sensor kinase (163). The roles of the AgrAC, ResDE, KdpED, LisRK, CesRK, LiaSR, and VirRS systems have been investigated in *L.*

monocytogenes (67, 169-178). The Agr system is involved in *L. monocytogenes* virulence in murine models, and in biofilm formation (67, 169, 170). ResD, the response regulator, controls respiration, repression of virulence gene expression and carbon source utilization (179). KdpED aids in the growth of *L. monocytogenes* during high osmotic conditions through regulation of the *kdp* locus that encodes for a high-affinity potassium uptake system

LisRK, CesRK, LiaSR and VirRS are TCSs that function in cell envelope stress response in *Listeria,* with LisRK and CesRK being the most extensively studied. VirRS controls virulence through modification of surface components (174). Genes under VirRS control include the *dlt*-operon and *mprF*, which are involved in D-alanylation of TAs and modification of phosphatidyl-glycerol in the membrane with L-lysine, respectively (174) (Table 1.2). LiaSR responds to cell wall active agents and regulates genes that are predicted to encode membrane or extracytoplasmic proteins and PBPA2 (177). Deletion of LiaS, the sensor kinase, resulted in an increased resistance to nisin, which is used in the food industry to inhibit the growth of *L. monocytogenes*, and an increase in cephalosporin sensitivity (176) (Table 1.2).

Table 1.2. Genes regulated by the cell-envelope stress response two-component systems.

Gene	Description of the gene product
LisRK Regulated Genes	
lmo0211	50S ribosomal protein L25/general stress protein Ctc
lmo0292	Similar to heat-shock protein *htrA* serine protease
lmo0802	Weakly similar to GTP-pyrophosphokinase
lmo1315	Similar to undecaprenyl diphosphate synthase
lmo1377	Two-component response regulator (LisR)
lmo1518	Conserved hypothetical protein.
lmo1680	Similar to cystathionine gamma-synthase
lmo1681	Similar to cobalamin-independent methionine synthase
lmo1690	Similar to membrane-bound metal-dependent hydrolase
lmo1698	Similar to ribosomal-protein-alanine *N*-acetyltransferase
lmo1919	Zn-dependent protease
lmo2210	Conserved hypothetical protein
lmo2350	Conserved hypothetical protein
lmo2522	Similar to cell-wall-binding protein
lmo2720	Similar to acetate-CoA ligase
CesRK Regulated Genes	
lmo0441	Penicillin-blinding protein class B (PBPB3)
lmo0443	Similar to transcription regulator of the LytR family
lmo1037	Integral membrane protein YoaT homologue
lmo1215	Similar to *N*-acetylmuramoyl-L-alanine amidase (autolysin)
lmo1416	Similar to glycopeptide antibiotics resistance protein
lmo2120	Similar to di-adenylate cyclase
lmo2420	Conserved hypothetical protein
lmo2442	Conserved hypothetical protein
lmo2687	Similar to cell division protein FtsW
lmo2688	Similar to cell division protein FtsW
lmo2689	Highly similar to Mg^{2+} transport ATPase

lmo2812	Similar to D-alanyl-D-alanine carboxypeptidase (PBPD2)
LiaSR Regulated Genes	
lmo0954	Conserved hypothetical protein
lmo0955	Phage-shock protein A homologue
lmo1966	Conserved hypothetical protein
lmo2229	Penicillin-binding protein class A (PBPA2)
lmo2482	*B. subtilis* YvlD homologue
lmo2485	Similar to stress-responsive transcription regulator
lmo2486	Conserved hypothetical protein
lmo2487	*B. subtilis* YvlB homologue
lmo2567	Conserved hypothetical protein
lmo2568	Conserved hypothetical protein
VirRS Regulated Genes	
lmo0972	D-alanine-poly(phosphoribitol) ligase subunit 2 (DltC)
lmo0973	D-alanyl transfer protein (teichoic acid) (DltB)
lmo0974	D-alanine-D-alanyl carrier protein ligase (DltA)
lmo2177	Conserved hypothetical protein

Table adapted from Nielson et al., (2012) and Fritsch et al. (2011) (173, 177).

LisRK is a positive regulator of LiaS, PBPA2 and HtrA, a serine protease (180, 181) (Table 1.2). Similarly to Δ*liaS*, deletion of the sensor kinase (Δ*lisK*) of *L. monocytogenes* LO28 resulted in enhanced resistance to nisin and sensitivity to cephalosporins (181). The mutant grew in 5% ethanol, a concentration that is bacteriostatic to wild type (182), but was not able to grow well in conditions of high osmolarity (BHI supplemented with 8% NaCl), suggesting a role for the TCS in osmoregulation (180). It was hypothesized that LisRK regulated genes are involved in altering membrane composition, because nisin, cephalosporins and ethanol all

affect membrane integrity (181). In addition, the mutant's tolerance of acidic conditions was growth phase-dependent; compared to the parental strain, Δ*lisK* was more tolerant of acidic environments during stationary phase, but was attenuated in virulence (182).

Similarly to LisRK, CesRK regulates virulence, ethanol tolerance, β-lactam sensitivity, and a PBP, PBPD2 (171-173) (Table 1.2). In addition, cell envelope-related genes (ex. PBPD2, autolysin) are part of the CesRK regulon (171, 173). In-frame deletion of *cesR* and *cesK*, the response regulator and sensor kinase, respectively, caused ethanol tolerance and decreased virulence, as demonstrated by decreased colonization in mouse models (172). In addition, the mutants were more sensitive to β-lactams including cephalosporins, but unaffected by antibiotics that affected protein synthesis (172). Using genome-wide transcriptional analysis, activation of genes under cefuroxime-induced conditions were found to be CesR- and LisR-dependent; these genes were also induced during intracellular infection. These results suggested that the TCSs involved in cell envelope stress could be activated during different stages of pathogenesis (173).

The only response regulator that affects *L. monocytogenes* biofilm formation is the orphan response regulator DegU (163). *L. monocytogenes* EGD-e Δ*degU* biofilm formation was ~40% of the parental strain and the cells were loosely adherent on plastic in rich media (163). In the absence

of DegU, flagellum synthesis is inhibited, resulting in non-motile cells. The mutant is less virulent, with 80% of mice alive after 10 days post-inoculation, whereas 100% of wild-type infected mice died by day 4 (163, 183, 184). Whether the other TCSs have a role in biofilm formation has yet to be determined.

Purpose and research aims

Many factors contribute to biofilm formation in *L. monocytogenes*, and understanding the developmental cycle of *L. monocytogenes* biofilms could lead to the identification of new antibiofilm strategies. The purpose of this study was to identify small molecules and enzymes that are able to modulate the *L. monocytogenes* biofilm formation cycle, and if possible, to determine their modes of action where they were unknown.

To identify molecules that affected *Listeria* biofilm development, a *Listeria* biofilm assay was developed for screening. The Z-factor (Z'), the screening window co-efficient, indicates the suitability of an assay for screening. It is a measurement of the difference between 3 standard deviations from the means of the positive and negative controls. An excellent assay is defined as $0.5 \leq Z < 1$ (185, 186). In this work, collections of eukaryotic kinase inhibitors and off patent FDA-approved bioactive molecules were screened for their effects on both growing and established biofilms.

The aims and findings of this study are discussed in the following three chapters:

1) Small molecule modulators of *Listeria monocytogenes* biofilm development**.**

This study describes the development of a *L. monocytogenes* biofilm assay suitable for high throughput screening. As proof of principle, a library of 80 eukaryotic protein kinase inhibitors was screened for their effects on biofilm development. Sphingosine and palmitoyl-D,L-carnitine are kinase inhibitors with a polar head group and saturated acyl chain that inhibited both *L. monocytogenes* and *S. aureus* biofilm formation. Sphingosine was bactericidal for both species at low micromolar concentrations, making it an interesting molecule for further investigation as an antimicrobial agent. Based on structure-activity relationship studies, we found that fatty acids alone had effects on biofilm formation that were dependent on chain length.

2) Role of PBPD1 in stimulation of *Listeria monocytogenes* biofilm formation by sub-minimal inhibitory β-lactam concentrations.

Using the biofilm assay that we established, a library of previously approved drugs with known mechanisms of action were screened for their

effects on biofilm formation and dispersal of pre-existing biofilms. A large number of hits were identified as β-lactams, which inhibited biofilm at high concentrations, but stimulated biofilm formation at sub-MIC levels. The stimulatory effect was independent of one of the TCS that functions in cell envelope stress response (CesRK) but required the target of the β-lactams, the PBPs. In the absence of PBPD1, biofilm stimulation was reduced in the presence of stimulatory β-lactams.

3) DNase and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms**.**

In this study, it was demonstrated that the EPS of *Listeria* is composed mainly of proteins and eDNA. Proteinase K dispersed biofilm grown on plastic and food-grade stainless steel effectively at low doses, whereas DNase was not as effective.

Together, my work demonstrates how small molecules and enzymes can be used as probes of *L. monocytogenes* biofilm formation and reveals aspects of biofilm formation that should be considered in order to design effective treatments for biofilm prevention and removal.

CHAPTER TWO

Small-molecule modulators of *Listeria monocytogenes* biofilm development

Co-authorship statement

Chapter Two consists of the following publication:

Nguyen UT, Wenderska IB, Chong MA, Koteva K, Wright GD, Burrows LL. 2012. Small-molecule modulators of *Listeria* monocytogenes biofilm development. Appl Environ Microbiol 78:1454-1465. Reproduced with permission from American Society of Microbiology.

The contributions of each author are outlined as follow:

1) The screening assay was developed by U.T.N. with input from I.B.W., G.D.W. and L.L.B.
2) MIC determination and biofilm assays using *L. monocytogenes* were conducted by U.T.N.
3) *S. aureus* biofilm assays were conducted by M.A.C.
4) K.K. synthesized and characterized carnitine derivatives.
5) The manuscript was conceived and written by U.T.N., K.K., G.D.W., and L.L.B.

Small molecule modulators of *Listeria* monocytogenes biofilm development

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Abstract

Listeria monocytogenes is an important food-borne pathogen whose ability to form disinfectant tolerant biofilms on a variety of surfaces presents a food safety challenge for manufacturers of ready-to-eat products. We developed here a high-throughput biofilm assay for *L. monocytogenes* and, as a proof of principle, used it to screen an 80-compound protein kinase inhibitor library to identify molecules that perturb biofilm development. The screen yielded molecules toxic to multiple strains of *Listeria* at micromolar concentrations, as well as molecules that decreased (≤50% of vehicle control) or increased (≥200%) biofilm formation in a dose-dependent manner without affecting planktonic cell density. Toxic molecules—including the protein kinase C antagonist sphingosine—had anti-biofilm activity at sub-minimal inhibitory concentrations. Structure-activity studies of the biofilm inhibitory compound palmitoyl-D,L-carnitine showed that while *Listeria* biofilm formation was inhibited with a 50% inhibitory concentration of 5.85 ± 0.24 μM, D,L-carnitine had no effect, whereas palmitic acid had stimulatory effects. Saturated fatty acids between $C_{9:0}$-$C_{14:0}$ were *Listeria* biofilm inhibitors, whereas fatty acids of $C_{16:0}$ or longer were stimulators, showing chain-length specificity. De novo-synthesized short-chain acyl carnitines were less effective biofilm inhibitors than the palmitoyl forms. These molecules, whose activities against bacteria have not been previously

established, are both useful probes of *L. monocytogenes* biology and promising leads for further development of anti-biofilm strategies.

Introduction

Among the key issues in the food industry is prevention of the proliferation of food-borne pathogens, including *Listeria monocytogenes*, on food contact surfaces and ready-to-eat products. Once ingested, *L. monocytogenes* can surmount three biological obstacles: the blood-brain barrier, the maternal-fetal barrier and the intestinal barrier (38, 187), leading to complications such as gastroenteritis, meningitis, still-birth or spontaneous abortions (32, 38, 188). In addition, *L. monocytogenes* can form mono- or multispecies biofilm communities on inert surfaces (44, 70). *L. monocytogenes* biofilms can be found in a variety of sites in food-processing facilities. The biofilms are highly resistant to ultraviolet light, desiccation, and sanitizing chemicals typically used for sterilization, providing opportunity for spread of *L. monocytogenes* to food (43, 49, 189). The addition of nisin, potassium/sodium lactate, and sodium acetate/diacetate to packaging material and/or food products to prevent the growth of *L. monocytogenes* has not eradicated infection, as demonstrated by the frequent recalls of *L. monocytogenes*-contaminated food products in North America (9, 79, 189-192), and outbreaks of listeriosis in Europe (32). To identify new ways of preventing food product

contamination, it is necessary to better understand the mechanisms underlying *L. monocytogenes* biofilm development.

A limited number of factors required for *L. monocytogenes* biofilm formation have been identified (recently reviewed by Renier et al. (66)). Biofilm development begins with initial attachment (reversible, then irreversible) to a surface, with the activation of genes involved in attachment, surface protein expression and extracellular polysaccharide (EPS) production. Further development from small microcolonies to mature biofilms is typically controlled by quorum sensing (65, 67, 170). Lastly, biofilm dispersal can result from shear forces, depletion of nutrients and accumulation of waste products. Degradation of the EPS matrix and/or upregulation of motility (62) allows dispersed cells to attach to a new site or existing biofilms to restart the cycle.

Many studies aimed at identifying pathways involved in biofilm formation have used genetic approaches, such as screening mutant libraries for those defective in biofilm formation (193-196). Although genetic approaches are useful, disadvantages include the difficulty of creating mutants in species not amenable to genetic manipulation and the under-representation of mutations in essential genes. The stresses imposed by some lesions can lead to downstream effects, including the accumulation of suppressor mutations (197, 198). In contrast, small molecules provide a way to conditionally inhibit (or stimulate) function—

even that of essential targets— over a range of concentrations, potentially providing novel insights into biological pathways. However, many small molecules inhibit more than a single cellular process (199), and identifying their targets can be challenging (200).

Given the number of potential pathways and genes contributing to biofilm development, we reasoned that use of small molecules as reagents to manipulate biofilm formation was warranted. Many studies have shown that it is possible to reduce the formation of food pathogen biofilms using specific small molecule food additives. In the presence of sodium levulinate, sodium lactate, or fatty acids, the growth of *L. monocytogenes* on ready-to-eat food was inhibited (189, 201). In addition, thyme essential oils, culinary herb extracts, or high molecular weight extracellular DNA can prevent adhesion of cells to a surface (46, 50, 202). Low concentrations of EDTA reduced initial cell attachment of *L. monocytogenes* to polyvinyl chloride without affecting planktonic cell density and inhibited cell-to-cell interactions (203). Quorum sensing in *L. innocua* was inhibited by natural compounds such as ambuic acid through repression of peptide biosynthesis (204). Thus, natural compounds or small molecules that target mechanisms involved in biofilm formation could be used to prevent their formation on food-contact surfaces.

In this work, we optimized a high-throughput biofilm assay for *L. monocytogenes* to make it suitable for small molecule screening, and as a

proof of principle, used it to test the effects of a collection of 80 eukaryotic protein kinase inhibitors on biofilm development. We reasoned that such molecules, which are largely based on chemical scaffolds that interact with the ATP-binding site of kinases and thus have the potential to interact with many proteins, may have unexpected activity in biofilm biology. We hypothesized that the use of molecules with known modes of action could provide useful clues to aid in identifying the targets of those with effects on *L. monocytogenes* biofilm formation.

Several molecules that altered biofilm development in a dose-dependent manner were identified, including the inhibitors sphingosine and palmitoyl-D,L-carnitine, both characterized by polar head groups and saturated 16-carbon acyl chains. Structure-activity studies using saturated fatty acids of defined acyl chain length showed that those from $C_{9:0}$ to $C_{14:0}$ were effective biofilm inhibitors with activity in the low micromolar range, while those from $C_{16:0}$ to $C_{18:0}$ stimulated biofilm formation. The inhibitory effects of select compounds on *L. monocytogenes* biofilm development on food-grade stainless steel were confirmed using scanning electron microscopy. We demonstrate that sphingosine and palmitoyl-D,L-carnitine also inhibit *Staphylococcus aureus* biofilm formation, showing that they have activity against other Gram-positive pathogens. These small molecules are useful tools for characterizing the process of *Listeria* biofilm

development and promising lead compounds for new antibiofilm strategies.

Materials and Methods

Bacterial strains and culture conditions

L. monocytogenes food isolates belonging to serotypes 1/2a and 1/2b were provided by Dr. Burton Blais of the Canadian Food Inspection Agency (CFIA; Ottawa, Ontario, Canada) and *L. monocytogenes* 568 (serotype 1/2a) was the gift of Dr. Lisbeth Truelstrup-Hansen (Dalhousie University). *S. aureus* 15981 was a gift from Dr. Julian Davies (University of British Colombia). The glycerol stocks of *L. monocytogenes* and *S. aureus* were stored at -80^{o}C prior to streaking them onto Difco tryptic soy agar (BD Biosciences), and LB-agar (BioShop), respectively and incubated at 37^{o}C overnight. After incubation, *L. monocytogenes* strains were used to inoculate 10 mL tryptic soy broth (TSB; EMD Chemicals) at 37^{o}C with agitation overnight. The overnight cultures were diluted in TSB to standardize the cultures to obtain an optical density at 600nm (OD_{600}) ~0.03 (Thermo Scientific BioMate™3). *S. aureus* 15981 was inoculated in 5 mL 66% TSB plus 0.2% dextrose overnight, with agitation at 37^{o}C. After incubation, the culture was standardized to an OD_{600} ~0.8 and subsequently diluted 1:200 in 25% TSB plus 0.2% dextrose prior to setting up the biofilm assay.

Preparation of test compounds

Compounds used for the present study were the Screen-Well™ kinase inhibitor library (ENZO Life Sciences), palmitoyl-D-carnitine hydrochloride (Crystal Chem Inc.), palmitoyl-D,L-carnitine hydrochloride, palmitoyl-L-carnitine hydrochloride, D,L-carnitine hydrochloride, myristoyl-D,L-carnitine hydrochloride, saturated fatty acids ($C_{9:0}$ – $C_{18:0}$) (all from Sigma-Aldrich), and ZM 449829 (Tocris). Stock solutions (≥10 mM in dimethyl sulfoxide [DMSO]; Caledon) were stored at -20°C and diluted in DMSO for the initial test concentrations (≤100 μM).

Determination of minimal inhibitory concentrations (MICs)

L. monocytogenes strains were inoculated overnight at 37°C in 10 mL TSB with agitation at 200 rpm. The overnight cultures were standardized to an OD_{600} of ~0.05 (4.3×10^7 CFU mL^{-1}) in TSB. *S. aureus* 15981 was inoculated in 5 mL of 66% TSB (2/3 strength of manufacturer's recommendation) plus 0.2% dextrose and then incubated overnight at 37°C and 200 rpm. After incubation, the culture was standardized to an OD_{600} of ~0.05 (5.7×10^6 CFU mL^{-1}) in 25% TSB plus 0.2% dextrose. The initial test concentrations of the compounds were diluted (1:100) in the culture (1 μl of compound in 99 μl of culture), and incubated at 37°C. The cultures were monitored at 24 h and 48 h, and the lowest concentration resulting in no growth after 48 h compared to the control samples was

defined as the MIC for *L. monocytogenes* 568, 1/2a, and 1/ 2b and *S. aureus* 15981. MIC determination did not follow the standard Clinical and Laboratory Standards Institute MIC guidelines because the cells did not grow in Mueller-Hinton broth (MHB), normally used for MIC determination, to an OD that was different from MHB sterility control.

L. monocytogenes and *S. aureus* biofilm assays

L. monocytogenes 568 was inoculated in 10 mL of TSB at 37^{o}C overnight, with shaking at 200 rpm, and subsequently standardized to an OD_{600} of ~ 0.03 in TSB. The initial test concentrations of the compounds were diluted (1:100) in standardized culture (1.5 μL of compound in 148.5 μL of culture). Control wells contained TSB plus 1% DMSO as a sterility control or standardized overnight culture plus 1% DMSO as a growth control. To prevent plate edge effects due to dehydration, the wells at the periphery of the plate were inoculated with 150 μL of sterile distilled H_2O (dH_2O). Biofilms were grown on polystyrene peg lids (Nunc), a method that produced more reproducible biofilms compared to using the surfaces of the wells. After placement of the peg lid, the plate was sealed with parafilm to prevent evaporation and incubated for 24 h at 37°C, 200 rpm. After 24 h of incubation, the planktonic growth was measured at OD_{600}, and the lid was transferred to a new microtiter plate with the same layout of TSB plus 1% DMSO, TSB with compounds, and water. The plate was

resealed with parafilm and incubated at 37°C, with shaking at 200 rpm, for 24 h. This step was repeated again for a total incubation time of 72 h (total of three passages, once every 24 h).

To quantify the amount of biofilms formed on the lid, the 96-peg lid was stained with crystal violet (CV) using a modified protocol (205). After 72 h, the lid was transferred to a new microtiter plate containing 200 μl of 1X phosphate-buffered saline (PBS) per well for 10 min to wash off any loosely adherent bacterial cells. After the wash step, the lid was transferred to a microtiter plate filled with 200 μL of 0.1% (wt/vol) CV for 15 min. To wash off excess CV, the lid was washed with 70 mL of dH_2O, in a single well tray, for 10 min. This step was repeated four times to ensure complete removal of excess CV. To solubilize the CV, the lid was transferred to a 96-well plate containing 200 μL of 95% ethanol or 33% (vol/vol) acetic acid per well for 15 min. The absorbance of the eluted CV was measured at 600 nm (BioTek ELx800).

The *S. aureus* 15981 biofilm assay was set up with the test compounds in the same manner as for the *L. monocytogenes* 568 assay, but biofilms were formed directly on the walls of each well of the 96-well plate. The control wells were filled with either (i) standardized culture plus 1% DMSO or (ii) 25% TSB plus 0.2% dextrose plus 1% DMSO, and then 150 μL of water was added to the wells at the periphery to prevent edge effects. The 96-well plate was covered with a MicroWell lid (Nunc), sealed

with parafilm, and incubated for 8 h, 37^{o}C, without agitation. After incubation and prior to staining of the biofilms, the OD_{600} of the planktonic culture was measured. The culture was removed from the wells, and the wells were washed with 200 μL of 1X PBS for 5 min. This step was repeated prior to staining the wells with 200 μL of 0.1% CV for 15 min. The wells were washed with excess dH_2O to remove unbound CV and air-dried in an inverted position for 30 min. Afterwards, 200 μL of 95% ethanol was added to the wells and incubated for 15 min at room temperature to elute bound CV, followed by measuring the absorbance of eluted CV at 600nm.

The planktonic density and CV absorbance data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc.). The IC_{50}s, defined as the half-maximal inhibitory concentration at which biofilm formation was inhibited by 50% compared to vehicle control, were calculated using GraphPad Prism 5 or GraFit 4 (Erithacus Software Ltd). Statistical values (P values) were calculated using a one-way analysis of variance (ANOVA) test and Dunnett's post test on GraphPad Prism.

Scanning electron microscopy of L. monocytogenes 568 on stainless steel

L. monocytogenes 568 biofilms were grown on food-grade, type 304H stainless-steel coupons (1 by 0.5 cm; Storm Copper Components Co.) in the absence (TSB plus 1% DMSO) or presence of select

compounds (sphingosine and palmitoyl-D,L-carnitine) at the indicated concentrations. Stainless steel coupons were placed in the wells of a 96-well plate with 200 μL of medium, and the plate was covered with a MicroWell lid. After 72 h (three passages, once every 24 h, as described above), the coupons were rinsed with PBS as described above and fixed in 2% glutaraldehyde (2% [vol/vol]) in 0.1 M sodium cacodylate buffer (pH 7.4; primary fixative) overnight. The coupons were then rinsed twice in buffer solution and postfixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. After the second fixation step, the samples were dehydrated through a graded ethanol series (50, 70, 70, 95, 95, 100, and 100%) and then transferred to the critical point dryer and allowed to dry. The coupons were mounted onto scanning electron microscopy (SEM) stubs, sputter coated with gold, and viewed under the VEGA/TESCAN LSU SEM. The images were acquired at 5,000x magnification using the VEGA/TESCAN software.

Synthesis of acyl carnitines

The synthesis of acyl carnitines was carried out using a modified version of the procedure described by Cervenka et al. (206). Briefly, 2.2 mM carbonylimidazole was added to a 2 mM solution of fatty acid in anhydrous toluene (1 mL). Activation was carried out until no more starting material could be detected using liquid chromatography-mass

spectrometry (LC/MS). Then, 2 mL of carnitine perchloride (prepared as described in the reference above) was added to the reaction mixture, followed by triethylamine (0.2 mL). The reaction was carried out for 1 to 2 days at 45°C. The progress of the reaction was monitored using LC/MS. At the end of the reaction time, 2 mL of methanol was added, and the solvent was removed under reduced pressure. The remaining oily residue was extracted twice with 5 mL of hexane. The hexane was discarded, and the remaining oil was dissolved in 1 mL of methanol and further extracted twice with 10 mL of hexane. The hexane was discarded, and the methanol was removed under vacuum. The remaining liquid was dissolved in 1 mL of 5% acetic acid and purified using a reverse-phase Sep-Pak cartridge. The final products were eluted with 5-mL aliquots of water-methanol, and the product's purity was confirmed using LC/MS, high-resolution mass spectrometry (HRMS), and one-dimensional (1D) and 2D nuclear magnetic resonance (NMR) experiments.

LC-ESI-MS analysis

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) data were obtained by using an Agilent 1100 Series LC system (Agilent Technologies Canada, Inc.) and a QTRAP LC/ MS/MS system (Applied Biosystems). Analytical reversed-phase high- pressure liquid chromatography was performed using a C18 column (Sunfire^{TM}; 5

μm, 4.6 by 50 mm; Waters) and a Agilent 1100 binary gradient pump system at a flow rate of 1 mL/min, under the following conditions: isocratic 5% solvent B (0.05% formic acid in acetonitrile) and 95% solvent A (0.05% formic acid in water) for 1 min, followed by a linear gradient to 97% solvent B over 7 min.

ESI experiments were performed on a using a Thermo Fisher LTQ-XL-Orbitrap Hybrid mass spectrometer (Thermo Fisher, Bremen, Germany), equipped with an electrospray interface operated in positive ion mode. Sample solution was directly infused into the mass spectrometer at a flow rate of 5 μL/min. The ESI source and MS parameters were automatically optimized and saved in a tune file for the base peak in the mass spectrum. Positive ESI source conditions included a sheath gas flow rate of 15 arbitrary units (AU), auxiliary gas flow rate of 5 AU, an ion spray voltage at 3.9 kV, a capillary temperature of 200°C, a capillary voltage of 23 V, and a tube lens voltage of 70 V. Normalized collision energy was 35%. Helium was used as the collision gas. The LTQ-XL-Orbitrap mass spectrometer experiment was set to perform a FT full scan from 100 to 2,000 *m/z* with the resolution set at 100,000 (at 500 *m/z*), followed by linear ion trap tandem MS (MS/MS) scans on the top three ions. Dynamic exclusion was set to 2, and selected ions are placed on an exclusion list for 30 s. The lock-mass option was enabled for the FT full scans using the

ambient air polydimethylcyclosiloxane (PCM) ion of *m/z* = 445.120024 or a common phthalate ion *m/z* = 391.284286 for real-time internal calibration.

1D and 2D NMR

1D (^{1}H and ^{13}C) and 2D NMR experiments (correlation spectroscopy, heteronuclear single-quantum correlation spectroscopy, and heteronuclear multiple-bond correlation spectroscopy) were carried out using a Bruker AVIII 700 MHz instrument in methanol-d4. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS) using the residual solvent signals at 3.30 and 49.00 ppm as internal references for the ^{1}H and ^{13}C spectra, respectively. The coupling constants (J) are reported in Hz.

Compound Data

(i) (3-Carboxy-2-nonanoyloxy-propyl)-trimethyl-ammonium

HRMS ES^{+}: $C_{16}H_{32}NO_4^{+}$, calc. 302.2326, found 302.2329. ^{1}H-NMR: 5.59 (m, 1H); 3.72 (m, 2H); 3.16 (s, 9H); 2.62 (dd, 1H, J1=4.4, J2= 4.04); 2.41 (dd, 1H, J1=J2= 9.6); 2.35 (m, 2H); 1.61 (p, 2H, J1=J2=J3=J4= 7.09); 1.31 (m, 10H), 0.96 (t, 3H, J1=J2=7.07). ^{13}C-NMR: 176.43; 174.12; 70.01; 67.82; 54.55; 35.23; 33.05; 30.37; 30.28; 30.22; 25.76; 23.70; 14.41.

(ii) (3-Carboxy-2-decanoyloxy-propyl)-trimethyl-ammonium

HRMS ES^+: $C_{17}H_{34}NO_4^+$, calc. 316.2482, found 316.2480. ^{1}H-NMR: 5.59 (m, 1H); 3.71 (m, 2H); 3.16 (s, 9H); 2.61 (dd, 1H, J1=4.1, J2= 4.2); 2.34-2.41 (m, 3H); 1.61 (p, 2H, J1=J2=J3=J4= 7.1); 1.30 (m, 12H), 0.89 (t, 3H, J1=J2=7.1). ^{13}C-NMR: 175.89; 174.13; 70.02; 67.93; 54.51; 35.22; 33.04; 30.56; 30.42; 30.22; 25.77; 23.74; 14.45.

(iii) (3-Carboxy-2-dodecanoyloxy-propyl)-trimethyl-ammonium

HRMS ES^+: $C_{19}H_{38}NO_4^+$, calc. 344.2795, found 344.2790. ^{1}H-NMR: 5.60 (m, 1H); 3.79 (m, 1H); 3.69 (m, 1H); 3.17 (s, 9H); 2.67 (dd, 1H, J1=J2= 4.4); 2.54 (dd, 1H, J1=J2= 8.5); 2.36 (t, 2H, J1=J2=7.6); 1.61 (p, 2H, J1=J2=J3=J4= 7.1); 1.29 (m, 16H), 0.89 (t, 3H, J1=J2=7.1). ^{13}C-NMR: 175.96; 174.15; 69.78; 67.17; 54.56; 35.17; 33.10; 30.73; 30.58; 30.46; 30.39; 30.19; 25.75; 23.74; 14.43.

Results

Identification of small-molecule modulators of *L. monocytogenes* biofilm formation

Systematic optimization of the medium and growth conditions for the *L. monocytogenes* biofilm assay resulted in Z' scores ≥ 0.60, making the assay suitable for high-throughput screening (185, 186). A high-quality assay is defined as $0.5 \leq Z < 1$ (185, 186). A pilot screen using the 80-compound Screen-Well™ kinase inhibitor library at an initial concentration

of 50 μM was performed in duplicate. We identified 23 compounds that reproducibly altered *L. monocytogenes* 568 planktonic cell density and/or biofilm development compared to the vehicle control (Table 2.1). Five compounds were planktonic growth inhibitors, fifteen compounds inhibited biofilm formation (defined as ≤50% of vehicle-treated control) but not planktonic cell density, and three compounds stimulated biofilm formation (≥200% compared to the vehicle-treated control) without affecting planktonic cell density (Table 2.1). The MICs of the planktonic growth inhibitors for *L. monocytogenes* 568 (serotype 1/2a), as well as food isolates belonging to serotypes 1/2a and 1/2b were determined. All three strains of *L. monocytogenes* had identical MICs for each test compound. Sphingosine had the lowest MIC of 12.5 μM, followed by rottlerin and tyrphostin 9 with MICs of 25 μM. Both GW 5074 and BAY 11-7082 had MICs of 50 μM. To determine whether the planktonic growth inhibitors had activity against other Gram-positive bacteria, they were tested against *S. aureus* 15981. Sphingosine, rottlerin and tyrphostin 9 had MICs of 12.5 μM, whereas both GW 5074 and BAY 11-7082 had MICs of 25 μM.

Inhibition of biofilm formation by planktonic growth inhibitors at sub-MIC concentrations

Compounds that were toxic in the initial screen were tested for their ability to inhibit biofilm formation at sub-MIC concentrations (Table 2.2). At

3.1 μM, a concentration that does not decrease planktonic cell density, sphingosine reduced biofilm formation to ~30% of control (Figure 2.1a). GW 5074 (IC_{50} of 3.78 ± 0.16 μM) and BAY 11-7082 (IC_{50} of 4.12 ± 0.27 μM) were more effective than tyrphostin 9 (IC_{50} of 4.77 ± 0.86 μM) at inhibiting *L. monocytogenes* 568 biofilm development. Whereas the planktonic cell density of GW 5074-treated cells increased up to 6.3 μM, then decreased beyond, the planktonic cell density of tyrphostin 9-treated cultures decreased slightly with increasing concentrations (see Figure S2.1 in the supplemental material). The planktonic cell density of BAY 11-7082 was unaffected at low concentrations (≤12.5 μM) (see Figure S2.1 in the supplemental material). Rottlerin was tested at sub-MIC concentrations, however the results were not reproducible (data not shown).

Effects of *L. monocytogenes* biofilm inhibitors

Seven of the most effective biofilm inhibitors (Table 2.2) and palmitoyl-D,L-carnitine (Table 2.3) identified in the initial screen were further tested in a dose-response assay. The compounds LFM-A13, SP 600125, ZM 449829, and Ro-318220 inhibited biofilm formation in a dose-dependent manner (see Figure S2.1 in the supplemental material; Table 2.2). SP 60012 (IC_{50} of 5.10 ± 0.36 μM) did not affect planktonic cell density, whereas a decrease in planktonic cell density occurred with

increasing concentrations of LFM-A13 (IC_{50} of 3.76 ± 0.16 μM) and Ro-318220 (IC_{50} of 22.1 ± 2.36 μM). Increasing concentrations of ZM 449829 (up to 50 μM) (IC_{50} of 5.57 ± 0.24 μM) resulted in a dose-dependent increase in planktonic cell density, but a decrease in biofilm formation (see Figure S2.1 in the supplemental material), which could indicate repartitioning of cells from the biofilm into the planktonic phase. Both biofilm development and planktonic growth were inhibited at 100 μM.

Indirubin-3'-monoxime inhibited biofilm formation in a dose-dependent manner without affecting planktonic cell density (IC_{50} of 22.3 ± 2.36 μM). However, at 3.1 μM (the lowest concentration tested) biofilm formation was stimulated (~140% compared to control) (see Figure S2.1 in the supplemental material). Similar results were seen with staurosporine where, at 3.1 μM, biofilm formation was stimulated (~160% compared to control). Increasing concentrations of staurosporine inhibited biofilm formation without affecting planktonic cell density (IC_{50} of 9.64 ± 1.95 μM). AG 879, which is structurally related to the planktonic growth inhibitor tyrphostin 9, was less effective at inhibiting biofilm formation (IC_{50} of 29.0 ± 4.04 μM). With increasing concentrations of AG 879, biofilm formation was reduced, while complete inhibition occurred at 100 μM, likely as a result of a corresponding decrease in planktonic cell density (see Figure S2.1 in the supplemental material).

Among the inhibitors identified in our initial screen was the acylated amino acid derivative, palmitoyl-D,L-carnitine, which is comprised of a long acyl-chain and polar head group, similar to sphingosine. Increasing concentrations of palmitoyl-D,L-carnitine (up to 50 μM) resulted in a dose-dependent reduction in biofilm formation with a concomitant increase in planktonic cell density (Table 2.3; Figure 2.2a), similar to the effect of ZM 449829. Even at the lowest concentration tested (3.1 μM), biofilm formation was ~60% of the control. At 100 μM, both biofilm formation and planktonic growth were inhibited. Because both palmitoyl-D,L-carnitine and sphingosine were effective biofilm inhibitors at sub-MIC concentrations and related structurally, we elected to further investigate their effects on biofilm formation.

Sphingosine inhibits *L. monocytogenes* biofilm formation in a concentration-dependent manner

Sphingosine inhibited *L. monocytogenes* biofilm formation on polystyrene at sub-MIC concentrations (Figure 2.1a). To determine whether the results were independent of the substratum on which biofilms were formed, *L. monocytogenes* biofilms were also grown on food-grade, type 304H stainless-steel coupons, with and without sphingosine. In the presence of 1% DMSO vehicle control (Figures 2.1b and c), many cells adhered to the stainless-steel surface, with some cells in multilayered

microcolonies. Interestingly, planktonic cell density of *L. monocytogenes* was not inhibited at the concentrations that were effective in the polystyrene peg-lid biofilm assay, even when the highest concentration tested was doubled to 25 μM (data not shown). However, in the presence of 3.1 μM sphingosine, the pattern of adherence to stainless steel was altered, with few cells near the air-liquid interface (Figure 2.1d), and more in regions where the coupons were submerged in medium (Figure 2.1e). In addition, there were no discernible microcolonies present and many of the cells appeared to be shorter or damaged compared to vehicle-treated controls. Although planktonic growth was not inhibited at 12.5 μM in this assay, there was a substantial reduction in the amount of cells adhering throughout the stainless steel coupons compared to the control samples (Figures 2.1f and g). Similar results were obtained with 25 μM (Figures 2.1h and i); very few cells attached to the coupons even though planktonic cell density was unaffected. Together these data show that sub-MIC concentrations of sphingosine reduce *L. monocytogenes* biofilm formation on both plastic and stainless-steel surfaces and that there are substratum-related differences with respect to its effective concentration.

Palmitoyl-D,L-carnitine inhibits biofilm formation

As shown in Figure 2.2a, biofilm formation on polystyrene was inhibited by palmitoyl-D,L-carnitine. To examine substratum-related effects,

biofilms were grown on stainless-steel coupons in the presence of various concentrations of palmitoyl-D,L-carnitine. At 6.3 μM, a reduced fraction of cells adhered to the stainless-steel coupons near air-liquid interface (Figure 2.2d) compared to the control samples (Figure 2.2b). In areas where the coupons were submerged in media, bacterial cell attachment appeared to be unaffected compared to the control (Figures 2.2c and e). In contrast to the polystyrene biofilm assay, where 25 μM palmitoyl-D,L-carnitine blocked biofilm formation, attachment on stainless-steel was comparable to the vehicle control in submerged areas (Figure 2.2g). At the air-liquid interface, few cells adhered to the surface compared to control samples (Figure 2.2f). At 50 μM, no adherent cells were detected on the stainless-steel coupons, either at the air-liquid interface or below (Figures 2.2h and i).

Structure-activity studies of palmitoyl-D,L-carnitine

Specific D-amino acids were recently reported to act as small molecule signals to induce dispersal of Gram-positive biofilms (207), with a range of effective concentrations from 3 μM (D-methionine) to 8.5 mM (D-leucine). Based on that report, we hypothesized that the D-carnitine component of palmitoyl-D,L-carnitine might be responsible for its biofilm-inhibitory activity. D,L-carnitine, palmitic acid, palmitoyl-D-carnitine and palmitoyl-L-carnitine were tested separately for their effects on *L.*

monocytogenes biofilm development. At concentrations where palmitoyl-D,L-carnitine inhibited biofilm development, neither planktonic cell density nor biofilm formation were affected by D,L-carnitine (Figure 2.3a). In contrast to palmitoyl-D,L-carnitine, which inhibited biofilm formation and increased planktonic cell density in a dose-dependent manner (Figure 2.2a), increasing concentrations of palmitic acid stimulated biofilm formation without significantly affecting planktonic cell density (≥ 25μM) (Figure 2.3b). Examination of enantiomer-specific inhibition of biofilm development by palmitoyl-D-carnitine and palmitoyl-L-carnitine showed that both compounds initially caused an increase in planktonic cell density at low micromolar concentrations, but the MIC for palmitoyl-D-carnitine was lower (25 μM) (Figure 2.3b) than that of palmitoyl-D,L-carnitine (Figure 2.2a) or palmitoyl-L-carnitine (Figure 2.3d), both at 100 μM. Interestingly, even though the stereochemistry of palmitoyl-carnitine affects its ability to inhibit planktonic growth, its effect on biofilm development does not appear to be enantiomer-specific, because both palmitoyl-D-carnitine and palmitoyl-L-carnitine inhibited biofilm formation to the same extent as the parent compound (Figures 2.3c and d). To examine the effect of acyl-chain length on activity, we synthesized additional acyl carnitines of specific chain length as described in the Materials and Methods and tested their effects on biofilm formation. Nonanoyl-D,L-carnitine and decanoyl-D,L-carnitine had minimal effects on *L. monocytogenes* biofilm formation

(Table 2.3). Lauroyl-D,L-carnitine and myristoyl-D,L-carnitine inhibited *L. monocytogenes* biofilm formation, but with reduced efficacy (IC_{50} of 10.1 ± 0.75 μM and 17.4 ± 2.13 μM, respectively) compared to palmitoyl-D,L-carnitine (IC_{50} of 5.85 ± 0.24 μM) (Table 2.3).

The effects of fatty acids on *Listeria* biofilm formation are chain-length specific

Because sphingosine and palmitoyl-D,L-carnitine had similar structures, with a charged head group coupled to a $C_{16:0}$ acyl chain, we further examined the effects of saturated fatty acids on biofilm formation. Although some fatty acids impair the growth of *L. monocytogenes* (201, 208), their effects on biofilm formation have not been reported. Saturated fatty acids with chain lengths ranging from $C_{9:0}$ to $C_{18:0}$ were tested for their effects on biofilm development (Table 2.4). The short-chain-length fatty acids $C_{9:0}$ and $C_{10:0}$ were less effective at inhibiting biofilm formation compared to medium-chain-length $C_{12:0}$, $C_{13:0}$, and $C_{14:0}$. Planktonic cell density was unaffected by short- or medium-chain fatty acids at the concentrations tested, whereas $C_{13:0}$ reduced growth at concentrations above 25 μM (data not shown). Similar to $C_{16:0}$, both $C_{17:0}$ and $C_{18:0}$ stimulated biofilm formation at concentrations ≥ 25 μM but did not change planktonic cell density relative to the vehicle control (data not shown).

Modulation of *S. aureus* biofilm formation by fatty acids and their derivatives

To determine whether the compounds identified as *Listeria* biofilm inhibitors had activity against other Gram-positive bacteria, specific compounds were tested against *S. aureus*. Both sphingosine and palmitoyl-D,L-carnitine were effective at reducing *S. aureus* growth and biofilm formation in a concentration-dependent manner (Figure 2.4). Sphingosine had a lower effective concentration against *S. aureus* (IC_{50} of 0.49 ± 0.01 μM) than against *L. monocytogenes* (IC_{50} of 2.81 ± 0.21 μM).

Palmitoyl-D,L-carnitine was less effective at inhibiting *S. aureus* biofilm formation compared to that of *L. monocytogenes* biofilm formation. As shown in Figure 2.4b, at low concentrations (1.6 μM to 6.3 μM), planktonic growth and biofilm formation were comparable to that of control. At 12.5 μM, planktonic cell density was reduced (~55% compared to the control), as was biofilm formation (~30% compared to control). Higher concentrations resulted in complete inhibition of *S. aureus* planktonic growth. When the constituents of palmitoyl-D,L-carnitine were tested separately, D,L-carnitine had no effect (Figure 2.3a), while $C_{16:0}$ stimulated biofilm formation at higher concentrations, similar to its effects on *Listeria* (≥25 μM) (Table 2.4; Figure 2.3b). When the effects of saturated fatty acids on *S. aureus* were tested, acyl chain length dependency was observed (Table 2.4). Fatty acids from $C_{9:0}$ to $C_{18:0}$ inhibited biofilm

formation; however, there was a decrease of at least 50% in planktonic cell density compared to the control at the highest concentration tested (100 μM), which was not observed for *L. monocytogenes* (data not shown). In contrast, $C_{16:0}$ to $C_{18:0}$ increased planktonic cell density with variable effects on *S. aureus* biofilm development. While $C_{16:0}$ induced biofilm formation at higher concentrations, $C_{17:0}$ and $C_{18:0}$ inhibited biofilm formation. In addition, myristoyl-D,L-carnitine had little effect on biofilm formation, suggesting that specific combinations of acyl chain length and head group affect the potency of the fatty acids and their derivatives against different species.

Discussion

L. monocytogenes biofilms are difficult to remove from industrial surfaces that may come into contact with ready-to-eat food products, leading to cross-contamination. We developed here an *L. monocytogenes* biofilm assay suitable for high-throughput screening and used it to identify small molecules that alter *L. monocytogenes* biofilm formation.

Similar to results reported for small molecule screens of *Pseudomonas aeruginosa* biofilms (205, 209), we identified both inhibitors and stimulators of biofilm formation. From our pilot screen of 80 compounds, 19% reduced biofilm formation and 4% increased biofilm development, compared to <1% of stimulators and biofilm inhibitors that

were identified from the screen of 66,095 compounds by Junker et al. (205). In a recent screen of the same 80-compound collection using *P. aeruginosa*, Wenderska et al. (209) found only two compounds (2.5%) that inhibited *P. aeruginosa* biofilm formation without affecting planktonic cell density. The differences in hit rates between screens may reflect the high density of known bioactives in the targeted kinase inhibitor library versus larger collections, and fewer efflux mechanisms in *L. monocytogenes* (210-212), compared with *P. aeruginosa* (213). Of note, the two compounds identified by Wenderska et al. as biofilm inhibitors were sphingosine and palmitoyl-D,L-carnitine, showing that these molecules have broad range antibiofilm activity against both Gram-negative and Gram-positive species. Although obvious homologues of the eukaryotic kinases are absent in prokaryotes, there are a number of potential targets, including histidine kinases belonging to two-component regulatory systems (214), nucleotide-binding proteins and/or phosphotransferases.

Many of the compounds identified as planktonic growth inhibitors also displayed biofilm inhibitory effects at sub-lethal doses (see Figure S2.1 in the supplemental material). Interestingly, two compounds that have dose-dependent biofilm inhibitory activity, staurosporine and indirubin-3'-monoxime, initially stimulated biofilm formation at low doses (see Figure S2.1 in the supplemental material). A similar result was reported previously for *Escherichia coli* and *P. aeruginosa*, where

subinhibitory concentrations of aminoglycosides stimulated biofilm formation (215).

Among the 20 compounds that were further tested in dose-response assays, sphingosine and palmitoyl-D,L-carnitine were selected for more detailed structure-function studies because they were potent and structurally similar inhibitors of biofilm formation. Sphingosine, derived from palmitoyl-CoA and serine, is an antimicrobial agent naturally produced on human skin, where it has been shown to prevent colonization by *S. aureus* (216, 217). It was previously reported to be an effective planktonic growth inhibitor of a variety of Gram-positive bacteria including – *L. monocytogenes* and *S. aureus* (216-219) – causing a 4-log reduction in planktonic cultures of *L. monocytogenes* at 25 μM (201). Our data are consistent with those studies, since concentrations above 6.3 μM inhibited *L. monocytogenes* planktonic growth in the polystyrene biofilm assay. Further, our data show that biofilm formation was impaired at sub-MIC concentrations. Notably, sphingosine was not an effective growth inhibitor when stainless-steel coupons were used as the substratum, even at concentrations that inhibited biofilm formation. This result suggests that exposure to stainless-steel surfaces or their eluates could interfere with the inhibitory property of the compound or modify bacterial physiology in a manner that allows growth even in the presence of increased inhibitor concentrations. This observation has important implications for the food

industry, since growth on stainless-steel surfaces may similarly promote increased resistance to other types of disinfectants. We also noted that there were more cells in submerged areas on the stainless-steel coupons in the presence of the compounds. This finding suggests that the target(s) are more highly expressed in cells exposed to aerobic conditions or that the compounds are more effective against rapidly growing cells at the air-liquid interface.

Sphingosine's protonated active form resembles that of quaternary ammonium compounds (219) that affect membrane integrity. Sphingosine is proposed to bind to the cell through electrostatic and hydrophobic interactions and to form pores that disrupt the cytoplasmic membrane (219). Sphingosine is also an inhibitor of protein kinase C (PKC), a family of enzymes involved in eukaryotic signal transduction pathways. It has been hypothesized a protein kinase analogous to that of mammalian cells may also be responsible for the antibacterial effect of sphingosine (201, 219), but this has yet to be experimentally demonstrated. As shown in Figures 2.1b-i, the integrity of attached *L. monocytogenes* cells appears to be compromised in the presence of increasing concentrations of sphingosine, even though planktonic cell density was not affected.

Palmitoyl-D,L-carnitine is also a palmitic-acid derived PKC inhibitor (220, 221). Palmitoyl-D,L-carnitine inhibited *L. monocytogenes* biofilm formation with a corresponding increase in planktonic cell density, possibly

due to repartitioning of the cells into the planktonic phase. This phenotype was different from that caused by sphingosine, suggesting that despite their structural similarities, the two molecules act via different mechanisms. *S. aureus* biofilm formation was also inhibited by palmitoyl-D,L-carnitine, supporting its broad anti-biofilm activity. Structure-activity studies showed that neither planktonic growth nor biofilm formation was affected by the D,L-carnitine component, a finding consistent with reports that L-carnitine is used by *L. monocytogenes* as an osmoprotectant in osmotic stress conditions (222-224). Because D,L-carnitine had no antibiofilm effect, we hypothesized the active component of palmitoyl-D,L-carnitine would be palmitic acid. Unexpectedly, palmitic acid stimulated biofilm development, for reasons that are not yet clear. Therefore, palmitoyl-D,L-carnitine has unique properties that transcend those of its constituents.

Based on the recent report of D-amino acids inducing biofilm dispersal (207), we tested palmitoyl-D-carnitine and palmitoyl-L-carnitine separately to determine whether the D-enantiomer was more effective. Palmitoyl-D-carnitine inhibited planktonic growth at 25 μM (Figure 2.3c) versus palmitoyl-L-carnitine and palmitoyl-D,L-carnitine which inhibit at 100 μM (Figures 2.3d and 2.2a, respectively), suggesting the D-form is a more potent *L. monocytogenes* growth inhibitor. However, the effects of palmitoyl-D,L-carnitine on biofilm formation are enantiomer-independent,

because the level of *L. monocytogenes* biofilm inhibition by all three compounds was similar. These data suggest that palmitoyl-D,L-carnitine may impair biofilm development through multiple mechanisms, as was reported for its effects on *P. aeruginosa* (209).

Based on their amphipathic structures, we also speculated that palmitoyl-D,L-carnitine and sphingosine might reduce bacterial attachment to surfaces via a surfactant or detergent-like effect. However, the length of the lipid tail makes the compounds more likely to form bilayers, rather than micelles, in an aqueous solution (225). To further test the surfactant hypothesis, we tested a variety of common laboratory detergents for antibiofilm activity, revealing that some detergents have inhibitory effects on biofilm formation with various effects on planktonic growth, while others had no effect (see Figure S2.2 in the supplemental material). Thus, detergent-like molecules, including palmitoyl-D,L-carnitine and sphingosine, can have biofilm-inhibitory activity that is independent of their surfactant properties.

Fatty acids have chain-length dependent antimicrobial activity against a variety of bacteria, but their effects on biofilm formation have been less well characterized. A number of recent studies have implicated microbially produced fatty acids or derivatives as diffusible signal factors that control, among other phenotypes, biofilm formation by heterologous species (226). Bovine milk, which contains a variety of fatty acids, has

been shown to reduce the amount of viable *L. monocytogenes* cells *in vitro*, as well as to prevent intestinal colonization of rats in a chain length-dependent manner (201, 208, 227). Sprong et al. showed that shorter-chain-length saturated fatty acids ($C_{4:0}$, $C_{6:0}$, and $C_{8:0}$) lack bactericidal activity toward *L. monocytogenes* even at 500 μM. In addition, neither $C_{16:0}$ nor $C_{18:0}$ had bactericidal activity at 500 μM, which is consistent with our results. In contrast, at 500 μM, long-chain unsaturated fatty acids ($C_{18:1}$ and $C_{18:2}$) and medium-chain-length saturated fatty acids ($C_{10:0}$, $C_{12:0}$, and $C_{14:0}$) reduced the number of viable cells (201, 227). Under our experimental conditions, $C_{9:0}$ to $C_{14:0}$ fatty acids did not reduce planktonic cell density, but were potent inhibitors of biofilm development (Table 2.4).

The structure-activity relationship of fatty acids and their derivatives is complex. Although palmitoyl-D,L-carnitine was a potent inhibitor of biofilm formation, short-chain acyl carnitines were less effective biofilm inhibitors than their free fatty acid equivalents (Tables 2.3 and 2.4). Sugar fatty acid esters were recently shown to inhibit biofilm formation *of L. monocytogenes* and other food-borne pathogens (228). Increasing sugar fatty ester chain length (>C_{12}) reduced the amount of *L. monocytogenes* biofilm formation, but the inhibition was less potent compared to the effects of the same molecules on *S. aureus* and *E. coli*. In contrast, a shorter-chain sugar fatty acid ester (C_8) did not inhibit biofilm formation

(208). Together these data suggest that the nature of fatty acid modification can significantly modulate effects on biofilm formation.

Biofilms are a major concern in the food industry since they can lead to contamination of food products. Some of the antibiofilm compounds identified through this work – including sphingosine and palmitoyl-D,L-carnitine – or their derivatives may have potential application against food-borne pathogens. Both inhibited both *L. monocytogenes* and *S. aureus* biofilm formation in the μM range and reduced the number of cells attaching to food-grade stainless steel and plastic, materials common in the food industry. These or related compounds can potentially be applied to equipment surfaces to prevent bacterial attachment or incorporated into food packaging to prevent bacterial growth. Identifying the mechanisms and targets involved in small-molecule modulation of *L. monocytogenes* biofilm formation can lead to biofilm inhibitors to be used alone or in conjunction with current sanitation methods used to prevent contamination.

Acknowledgements

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(CFIA). Notwithstanding, the CFIA does not necessarily endorse the views or practices presented by the authors in this manuscript. I.B.W. was the recipient of a Cystic Fibrosis Canada Studentship, and M.A.C. was the recipient of CFC Summer Studentship awards in 2010 and 2011.

Figure 2.1

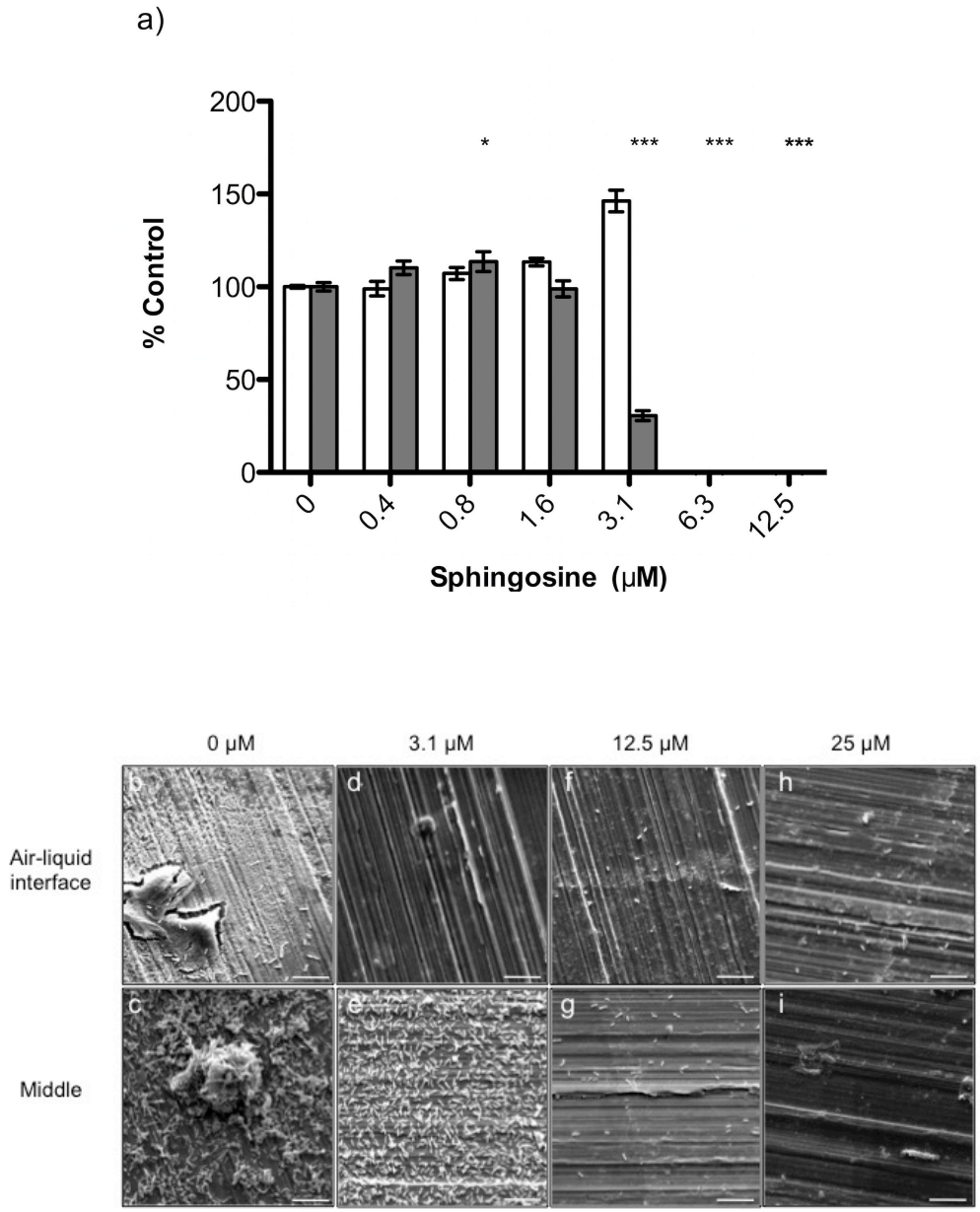

a)
200
150
100
50
0
% Control
*
***
***
***
0
0.4
0.8
1.6
3.1
6.3
12.5
Sphingosine (μM)
0 μM
3.1 μM
12.5 μM
25 μM
Air-liquid interface
Middle
b
d
f
h
c
e
g
i

Figure 2.1. Inhibition of biofilm formation on different surfaces by sphingosine. (a) *L. monocytogenes* biofilms were grown on polystyrene pegs and quantified using crystal violet staining. Planktonic growth at day 3 (white bars) and biofilm formation (grey bars), expressed as a percentage of control (n=4, with standard errors shown). *, $P<0.05$; **, $P<0.01$; ***, $P< 0.001$ (b-i) Representative SEM images of *L. monocytogenes* grown on food-grade stainless-steel coupons in the presence or absence of sphingosine at various concentrations. Images were captured near the air-liquid interface and middle of coupons, where they were submerged in media. Bar: 10 μm. Magnification: 5000x.

Figure 2.2

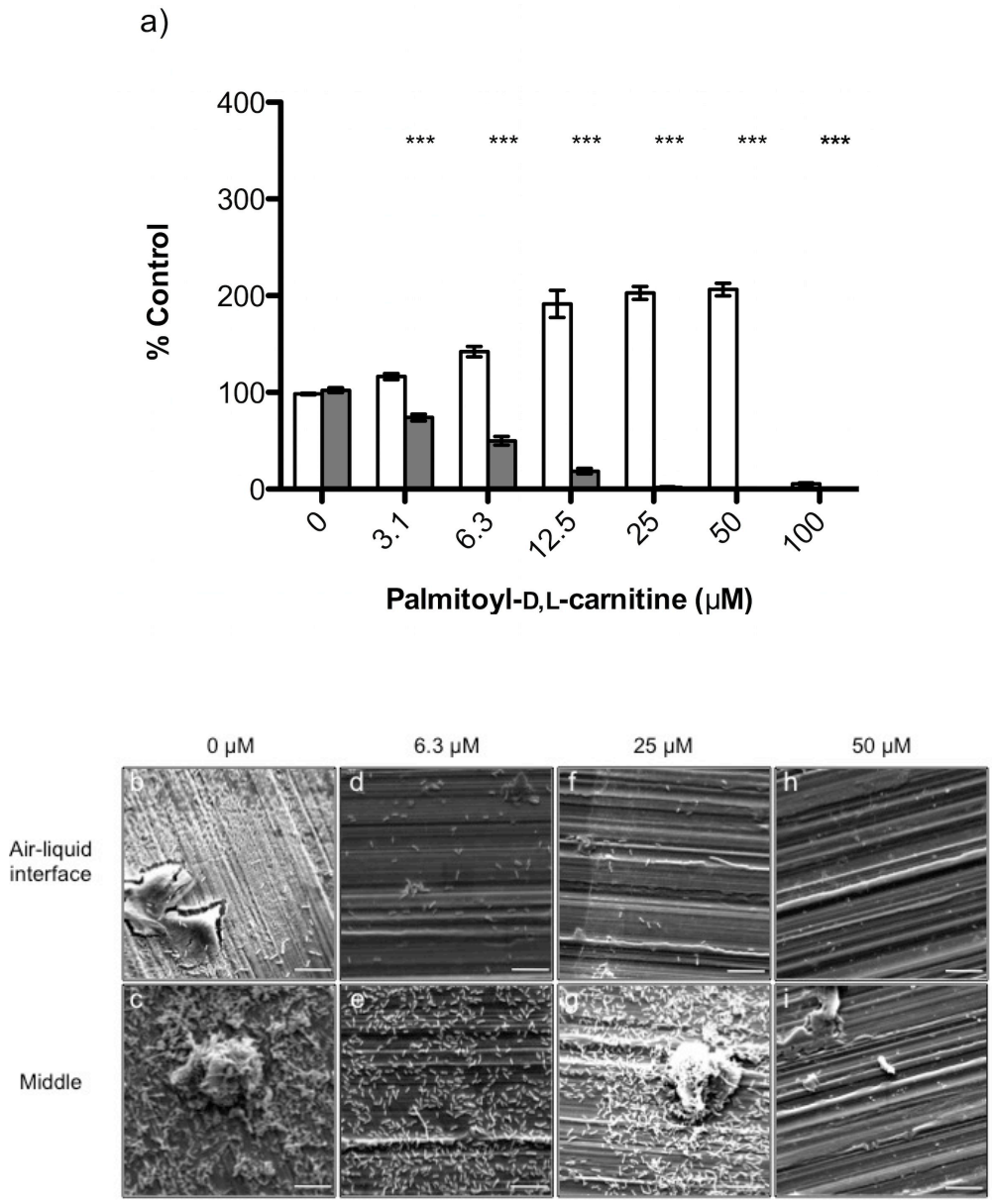

a)
400
300
200
100
0
% Control
***
***
***
***
***
***
0
3.1
6.3
12.5
25
50
100
Palmitoyl-D,L-carnitine (μM)
0 μM
6.3 μM
25 μM
50 μM
Air-liquid interface
Middle
b
d
f
h
c
e
g
i

Figure 2.2. Palmitoyl-D,L-carnitine inhibits *L. monocytogenes* biofilm formation. (a) *L. monocytogenes* biofilms were grown on polystyrene pegs using the microtiter assay and quantified with crystal violet staining. Planktonic growth at day 3 (white bars) and biofilm formation (grey bars) expressed as a percentage of control (n=4 with standard errors shown). ***, *P*< 0.001 (b-i) Representative SEM images of *L. monocytogenes* grown on food-grade stainless-steel coupons in the absence (1% DMSO) or presence of palmitoyl-D,L-carnitine. Images captured near the air-liquid interface and areas where coupons were submerged in media.
Bar: 10 μm. Magnification: 5000x.

Figure 2.3

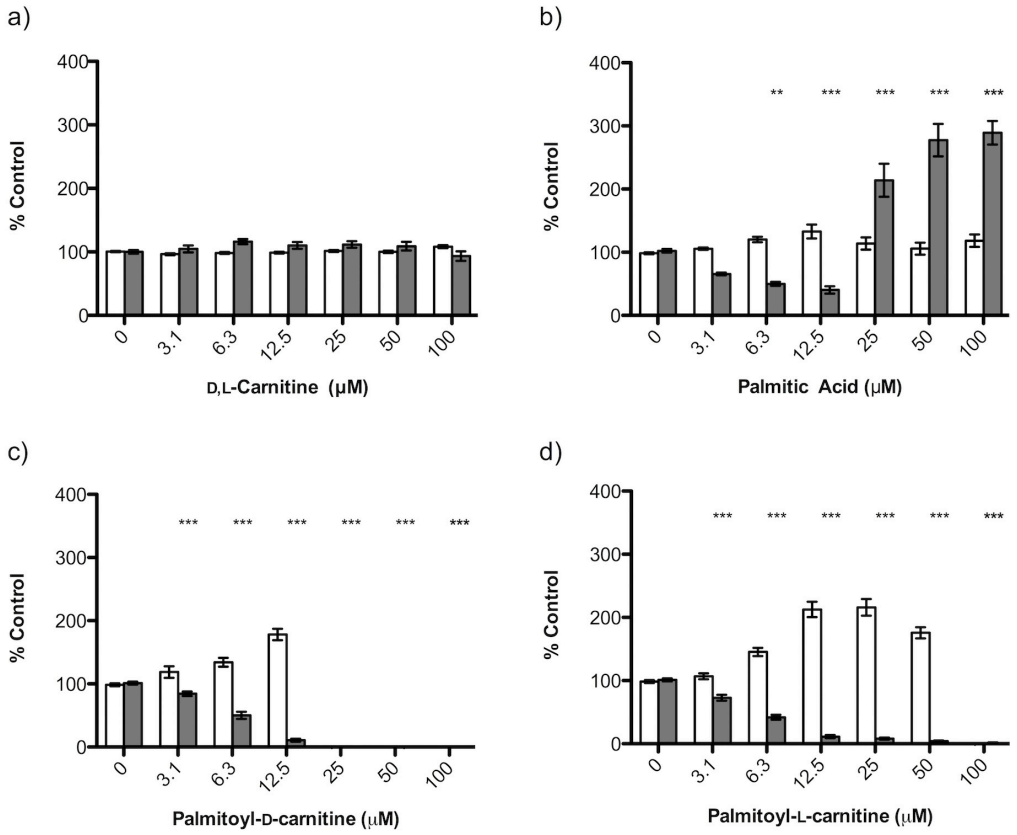

a)
b)
c)
d)
% Control
400
300
200
100
0
0
3.1
6.3
12.5
25
50
100
D,L-Carnitine (µM)
Palmitic Acid (µM)
Palmitoyl-D-carnitine (µM)
Palmitoyl-L-carnitine (µM)
**
***

Figure 2.3. Structure-activity studies of palmitoyl-D,L-carnitine.

L. monocytogenes biofilms were grown on polystyrene pegs in the presence of D,L-carnitine (a), palmitic acid (b), palmitoyl-D-carnitine (c), or palmitoyl-L-carnitine (d). The amount of biofilm formed was quantified by crystal violet staining. Planktonic growth at day 3 (white bars) and biofilm formation (grey bars) are expressed as a percentage of control. (n=4). *, $P<0.05$; **, $P<0.01$; ***, $P< 0.001$.

Figure 2.4

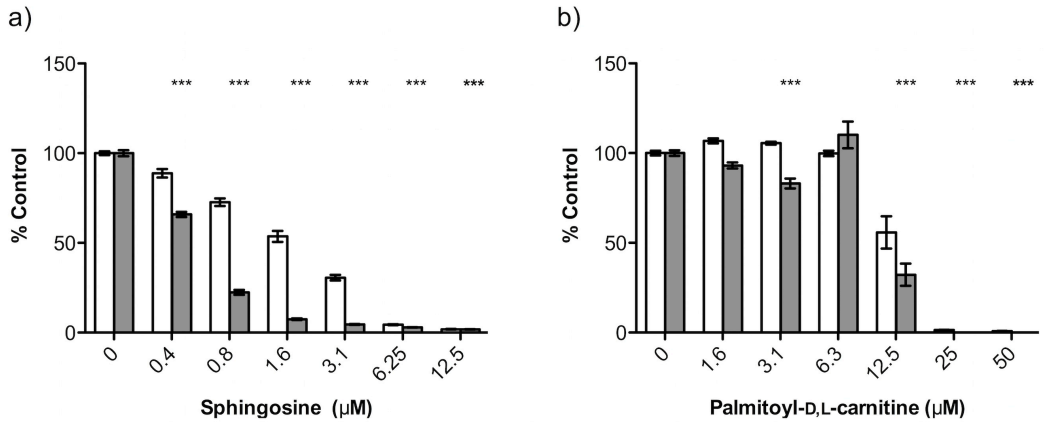

a)
b)
% Control
150
100
50
0
***
0
0.4
0.8
1.6
3.1
6.25
12.5
Sphingosine (μM)
1.6
3.1
6.3
12.5
25
50
Palmitoyl-D,L-carnitine (μM)

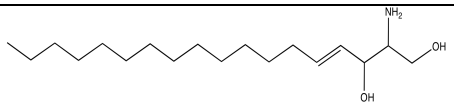
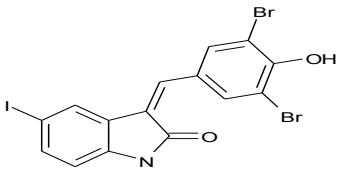
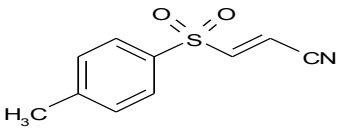
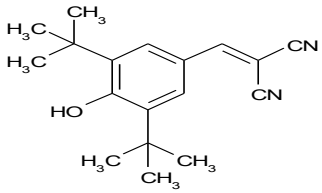
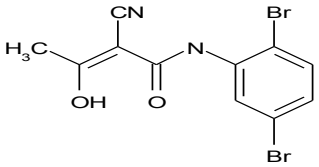
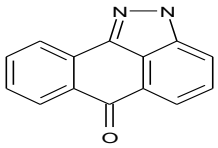
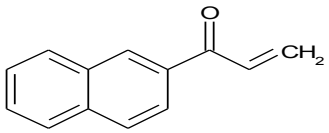
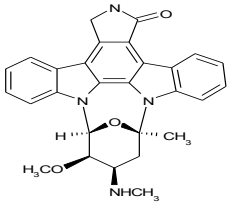
Figure 2.4. *S. aureus* biofilm development is inhibited by sphingosine and palmitoyl-D,L-carnitine. *S. aureus* biofilms were grown in microtiter plates in the presence of either (a) sphingosine or (b) palmitoyl-D,L-carnitine. Crystal violet staining was used to quantify the amount of biofilm formed. Planktonic growth (white bars) and biofilm formation (grey bars) are expressed as a percentage of control samples (n=3). ***, $P < 0.001$.

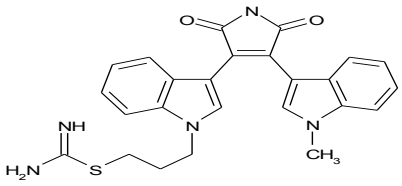
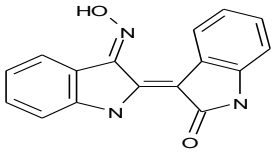
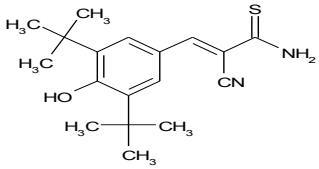
Table 2.1. Compounds that modulate *L. monocytogenes* biofilm development

Planktonic Growth Inhibitors[a]	Biofilm Inhibitors[b]	Biofilm Stimulators[c]
GW 5074	U-0126	Kenpaullone
Tyrphostin 9	LFM-A13	KN-62
Sphingosine	SB-202190	PKC-412
Rottlerin	BML-257	
BAY 11-7082	AG-490	
	AG-879	
	ZM 449829	
	KN-93	
	Staurosporine	
	Hypericin	
	SP 600125	
	Ro 31-8220	
	Palmitoyl-DL-carnitine	
	Indirubin	
	Indirubin-3'-monoxime	

[a] Planktonic growth inhibitors were defined as compounds that reduced growth by ≤50% of vehicle control growth at the initial concentration of 50μM.
[b] Biofilm inhibitors were identified as compounds that reduced biofilm formation by ≤50% of vehicle control without affecting planktonic cell density.
[c] Biofilm stimulators were defined as those compounds that increased biofilm formation by ³200% as compared to vehicle control without affecting planktonic cell density.

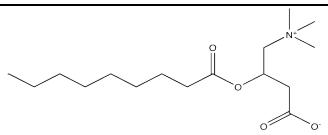
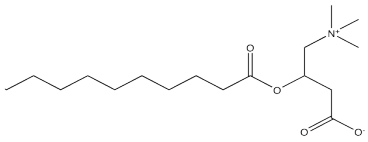
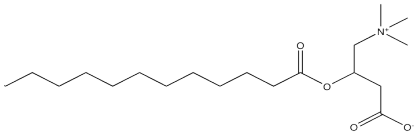
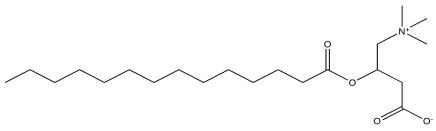
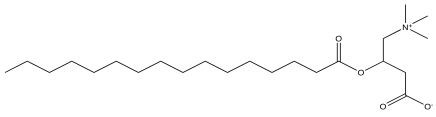
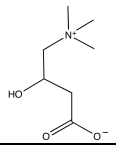
Table 2.2. Half-maximal inhibitory concentrations of planktonic growth inhibitors and biofilm inhibitors on biofilm formation

Test Compound	Structure	*L. monocytogenes* IC_{50} (μM)[a]
Sphingosine		2.81 ± 0.21
GW 5074		3.78 ± 0.16
BAY 11-7082		4.12 ± 0.27
Tyrphostin 9		4.77 ± 0.86
LFM-A13		3.76 ± 0.16
SP 600125		5.10 ± 0.36
ZM 449829		5.57 ± 0.24
Staurosporine		9.64 ± 1.95

Ro 31-8220		22.1 ± 2.36
Indirubin-3'-monooxime		22.3 ± 2.43
AG-879		29.0 ± 4.04

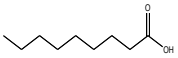
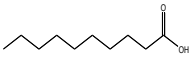
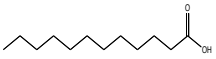
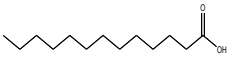
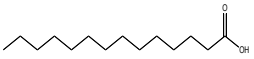
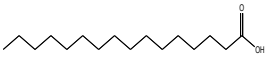
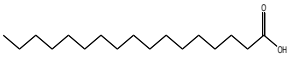
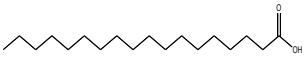
[a] That is, concentrations at which biofilm formation was inhibited by 50% compared to vehicle control.

Table 2.3. Half-maximal inhibitory concentrations of carnitine and acylcarnitines on biofilm formation

Test Compound	Structure	*L. monocytogenes* IC_{50} (μM)[a]
Nonanoyl-D,L-carnitine		57.1 ± 9.61*
Decanoyl-D,L-carnitine		78.6 ± 21.2*
Lauroyl-D,L-carnitine		10.1 ± 0.75
Myristoyl-D,L-carnitine		17.4 ± 2.13
Palmitoyl-D,L-carnitine		5.85 ± 0.24
D,L-carnitine		Minimal effect

[a] That is, the concentrations at which biofilm formation was inhibited by 50% compared to vehicle control samples.
*, Non-ideal behaviour, since the data does not go to 0% of the control at highest concentration tested (50 μM)

Table 2.4. Half-maximal inhibitory concentrations of fatty acids on biofilm formation

Test Compound	Structure	IC_{50} (μM)[a]	
		L. monocytogenes	***S. aureus***
$C_{9:0}$		33.2 ± 4.67	4.75 ± 1.24
$C_{10:0}$		20.8 ± 2.11	7.81 ± 1.01
$C_{12:0}$		4.10 ± 0.27	5.81 ± 0.78
$C_{13:0}$		4.34 ± 0.23	6.53 ± 0.39
$C_{14:0}$		2.50 ± 0.26	6.38 ± 0.08
$C_{16:0}$		3.39 ± 1.95	Stimulates[b]
$C_{17:0}$		Stimulates[b]	13.8 ± 4.74
$C_{18:0}$		Stimulates[b]	4.02 ± 1.81

[a] That is, concentration at which biofilm formation was inhibited by 50% compared to vehicle control samples.
[b] "Stimulates" indicates that the IC_{50} was not detected at the concentrations tested; biofilm formation was >150% at the highest concentration tested (100 μM) compared to vehicle control samples.

Figure S2.1

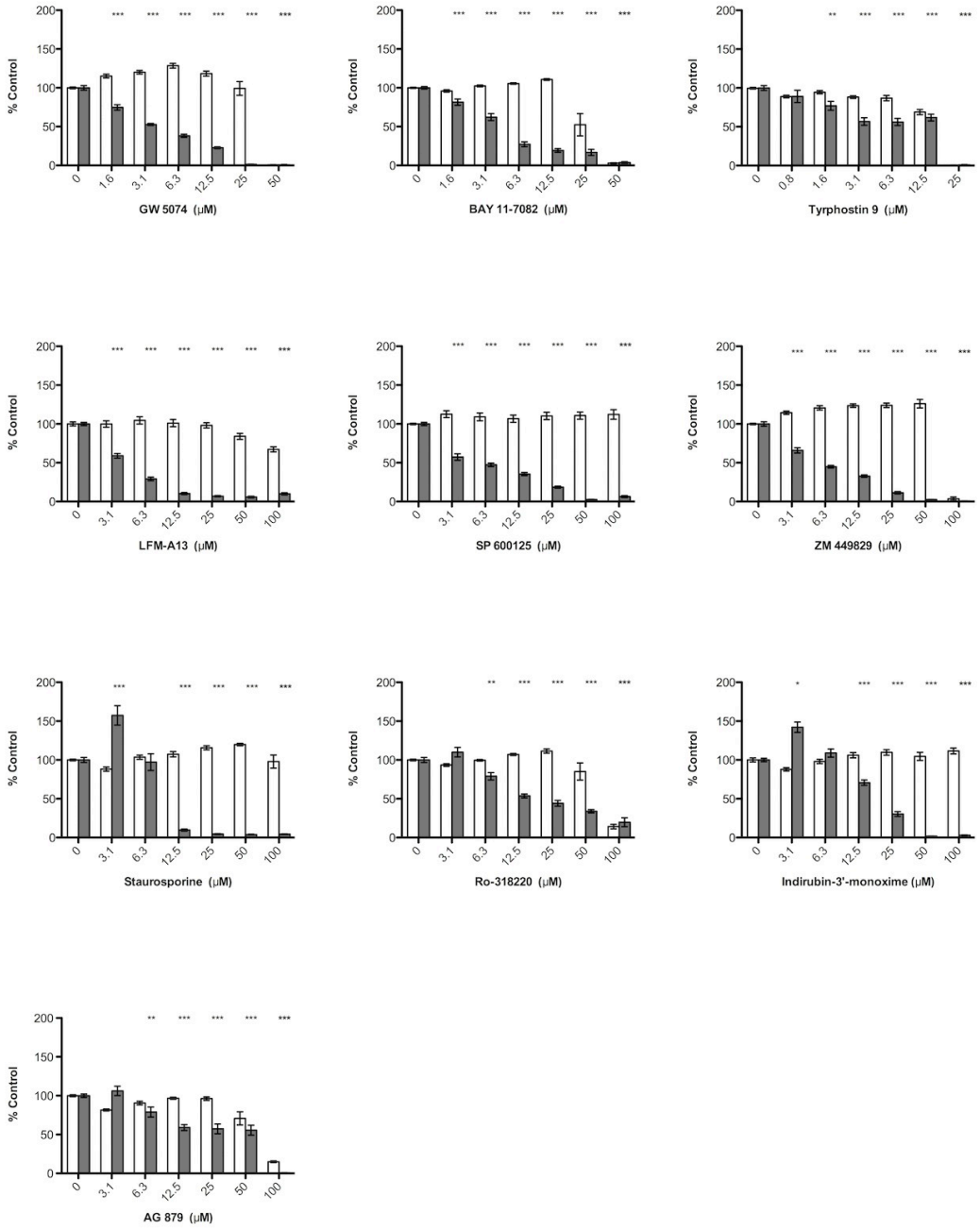

% Control
GW 5074 (μM)
BAY 11-7082 (μM)
Tyrphostin 9 (μM)
LFM-A13 (μM)
SP 600125 (μM)
ZM 449829 (μM)
Staurosporine (μM)
Ro-318220 (μM)
Indirubin-3'-monoxime (μM)
AG 879 (μM)

Figure S2.1. The effects of planktonic growth inhibitors and biofilm inhibitors on biofilm formation. *L. monocytogenes* biofilms were grown on polystyrene pegs and quantified using crystal violet staining. Planktonic growth at day 3 (white bars) and biofilm formation (grey bars), expressed as a percentage of control samples (n=3). *, $P<0.05$; **, $P<0.01$; ***, $P< 0.001$.

Figure S2.2

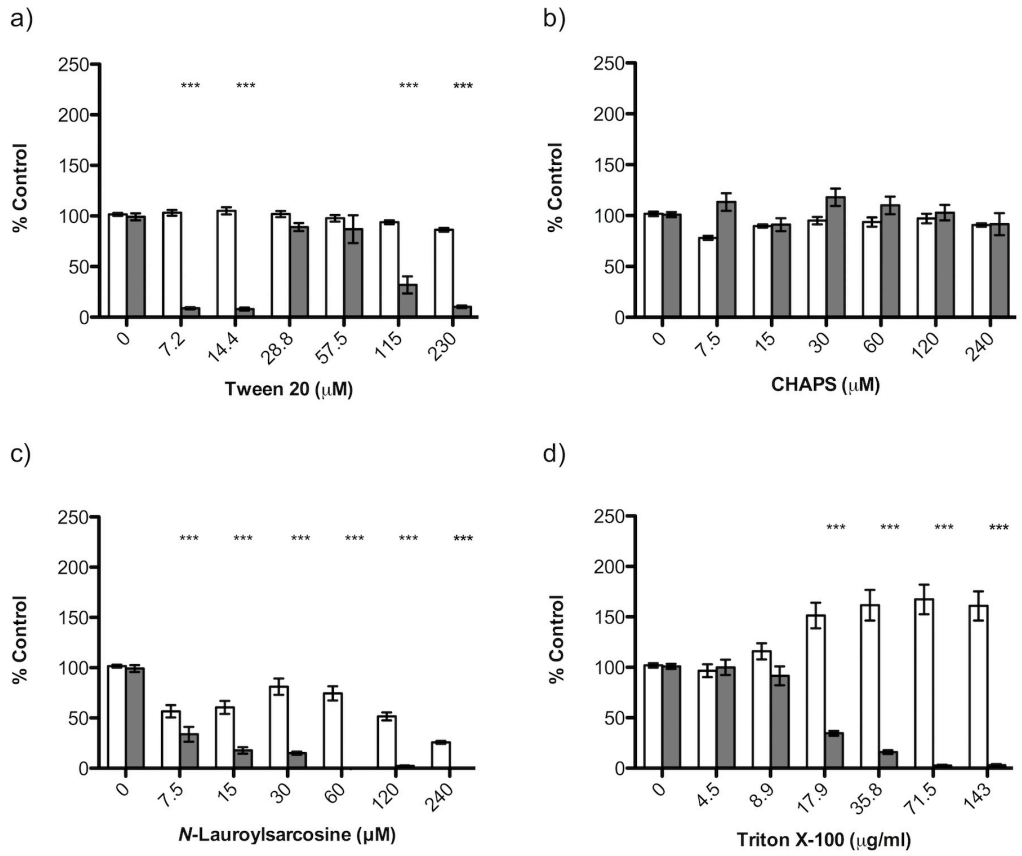

a)
b)
c)
d)
% Control
250
200
150
100
50
0
***
0
7.2
14.4
28.8
57.5
115
230
Tween 20 (μM)
7.5
15
30
60
120
240
CHAPS (μM)
N-Lauroylsarcosine (μM)
4.5
8.9
17.9
35.8
71.5
143
Triton X-100 (μg/ml)

Figure S2.2. The effects of various detergents on biofilm formation. *L. monocytogenes* biofilms were grown on polystyrene pegs and quantified using crystal violet staining. Planktonic growth at day 3 (white bars) and biofilm formation (grey bars), expressed as a percentage of control samples (n=4). ***, $P< 0.001$.

CHAPTER THREE

Role of PBPD1 in stimulation of *Listeria monocytogenes* biofilm formation by sub-minimal inhibitory β-lactam concentrations

Chapter Three- Contributions of the authors' statement

Chapter Three consists of the following publication:

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The contributions of each author are outlined as follow:

1) The biofilm dispersal screen and follow up biofilm assays were conducted by U.T.N.
2) The initial biofilm inhibitory screen was conducted by A.J.H.
3) CesK primers were designed by A.C.F.A.
4) H.H. constructed PBPD1 and CesK mutants.
5) Whole cell lysate and membrane PBP profiling were conducted by U.T.N and A.C.F.A, respectively
6) Manuscript was conceived and written by U.T.N. H.H, G.D.W, and L.L.B

Role of PBPD1 in stimulation of *Listeria monocytogenes* biofilm formation by sub-minimal inhibitory β-lactam concentrations

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Running title: *Listeria* biofilms and sub-MIC β-lactams

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Abstract

Disinfectant-tolerant *Listeria monocytogenes* biofilms can colonize surfaces that come into contact with food, leading to contamination and potentially, food-borne illnesses. To better understand the process of *L. monocytogenes* biofilm formation and dispersal, we screened 1120 off-patent FDA-approved drugs and identified several that modulate *Listeria* biofilm development. Among the hits were more than 30 β-lactam antibiotics, with effects ranging from inhibiting (≤50%) to stimulating (≥ 200%) biofilm formation compared to control. Most β-lactams also dispersed a substantial proportion of established biofilms. This phenotype did not necessarily involve killing, as >50% dispersal could be achieved with concentrations as low as 1/20 of the minimal inhibitory concentration (MIC) of some cephalosporins. Penicillin-binding protein (PBP) profiling using a fluorescent penicillin analogue showed similar inhibition patterns for most β-lactams, except that biofilm-stimulatory drugs did not bind PBPD1, a low molecular weight D,D-carboxypeptidase. Compared to wild type, a *pbpD1* mutant had an attenuated biofilm response to stimulatory β-lactams. The cephalosporin-responsive CesRK two-component regulatory system, whose regulon includes PBPs, was not required for the response. The requirement for PBPD1 activity for β-lactam stimulation of *L. monocytogenes* biofilms shows that the specific set of PBPs that are

inactivated by a particular drug dictates whether a protective biofilm response is provoked.

Introduction

L. monocytogenes biofilm formation is a complex process that involves changes in transcriptional regulation, metabolism, flagellum and peptidoglycan biosynthesis (14, 48, 112). Although a number of genes with potential roles in biofilm development in other bacteria have been identified, those involved in *L. monocytogenes* biofilm formation are not well characterized. The first committed step of biofilm formation is irreversible attachment to a surface, followed by the production of a protective extracellular polymeric substance (EPS) matrix and development into a mature biofilm. This process is controlled by quorum sensing, used for cell density-dependent communication (63, 229). The EPS surrounds the cells, helping them to withstand environmental stresses. In *L. monocytogenes*, the EPS matrix contains proteins and nucleic acids, but no polysaccharides have been identified, and sequenced genomes lack genes encoding polysaccharide biosynthetic machinery (46, 70). Upon upregulation of motility, EPS matrix degradation, or depletion of oxygen, mature biofilms can disperse into smaller aggregates or individual planktonic cells, reinitiating biofilm formation (44,

62, 74). To prevent transmission of *L. monocytogenes*, it is important to better understand the process of biofilm development and dispersal.

Once ingested by a susceptible host, *L. monocytogenes* can cross three biological barriers: the blood-brain, intestinal, and maternal-fetal barriers and potentially cause septicaemia, encephalitis, gastroenteritis or spontaneous abortion (38, 187). In the intestinal tract, the pathogen can form biofilm-like aggregates in the cecum and colon, allowing it to persist (36). There are currently no vaccines and few effective antibiotics for *L. monocytogenes* infections (31). The first line of treatment for infected individuals is typically β-lactam antibiotics, specifically penicillins (e.g. ampicillin) which target the penicillin-binding proteins (PBPs) that play key roles in peptidoglycan (PG) biosynthesis and remodelling (230). Penicillins are sometimes used in combination with aminoglycosides for 14 – 21 d (31, 40). In contrast, cephalosporins are not typically used to treat listeriosis, as *L. monocytogenes* is naturally resistant, with high minimal inhibitory concentrations (MICs) (138, 231).

PG is composed of alternating sugar repeats of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). Each NAM residue is substituted with a pentapeptide (stem peptide) terminating in D-Ala-D-Ala. The stem peptide can be cross-linked to an adjacent stem peptide, often through a peptide bridge in Gram positives, thereby linking the glycan chains (123, 124). The PG of Gram-positive *L. monocytogenes*

is unusual in that it resembles that of Gram-negative bacteria, with diaminopimelic acid at the 3rd position of the stem peptide, and direct cross-links between stem peptides, without an intervening peptide bridge (121). PBPs are divided into three classes: high-molecular weight (HMW) class A, HMW class B and low-molecular-weight (LMW) PBPs (127). Class A HMW PBPs have N-terminal transglycosylase and C-terminal transpeptidase domains, while class B HMW PBPs have C-terminal transpeptidase domains coupled to N-terminal domains of unknown function (127). The transglycosylase domain links disaccharide-pentapeptide PG subunits to existing PG, forming the β-1,4-glycosidic linkage between the sugars, while the transpeptidase domain crosslinks the stem peptides (121, 123, 129, 130). LMW PBPs have D,D-carboxypeptidase activity – which removes the terminal D-Ala from the stem peptide to control the extent of crosslinking – and/or endopeptidase activity, which cleaves the cross-linked peptides (121, 127, 130-132). β-lactams mimic the structure of the D-Ala-D-Ala moiety of the stem peptide and inhibit the transpeptidase and carboxypeptidase activities of PBPs via covalent modification of the critical active-site serine. Further cross-linking of stem peptides is thus prevented, leading to loss of PG integrity and eventually, cell death (124, 131, 133, 232).

Here we used a previously developed *L. monocytogenes* biofilm assay (59) to screen a library of 1120 previously FDA-approved, off-patent

drugs, to identify molecules that modulate *L. monocytogenes* biofilm formation, prevent biofilm development and/or disperse established biofilms. Since many of these drugs have known modes of action, they represent useful probes for identifying targets that affect biofilm formation. Many β-lactams – those commonly prescribed to treat listeriosis, as well as those to which *L. monocytogenes* is considered resistant – inhibited biofilm formation at higher concentrations, but stimulated biofilm formation (>200% of control) at concentrations well below the minimal inhibitory concentration (MIC). The stimulatory effects of sub-MIC β-lactams required LMW PBPD1 but not CesRK, a two-component system activated by exposure to cephalosporins (171, 172). These data suggest that the specific subset of PBPs that are targeted by sub-MIC β-lactams dictates whether a protective biofilm response is induced.

Materials and Methods

Bacterial strains and plasmids

L. monocytogenes food isolates of serotypes 1/2a and1/2b were the gift of Dr. Burton Blais of the Canadian Food Inspection Agency (CFIA - Ottawa, Ontario). *L. monocytogenes* 568 and EGD-e (serotypes 1/2a) and the temperature sensitive plasmid used for mutagenesis, pAUL-A (233), were the kind gift of Dr. Lisbeth Truelstrup-Hansen (Dalhousie U.). Glycerol stocks of *L. monocytogenes* were stored at -80^{o}C prior to

streaking them onto Difco tryptic soy agar (BD Biosciences) for the biofilm assays or brain heart infusion (BHI) agar for construction of the *L. monocytogenes* mutants. *Escherichia coli* DH5α was stored at -80^{o}C prior to electroporation of deletion constructs.

Preparation of test compounds

The 1120 compounds, referred to as the Previous Approved Drug or PAD library (234) were initially screened at a concentration of 10 μM (stock at 1 mM in OmniSolv® dimethlysulfoxide (DMSO); EMD). β-lactams were purchased from Sigma-Aldrich and stock solutions (≥1mM in DMSO) were stored at -20^{o}C. Stock solutions were then diluted in DMSO for the initial test concentration ranges before incubation with bacterial culture in TSB, final concentration 1% DMSO.

Minimal inhibitory concentration (MICs) determinations

Determination of the MICs for *L. monocytogenes* strains was performed as previously described (59). *L. monocytogenes* does not grow in Mueller Hinton Broth (MHB), the standard medium for MIC determination according to Clinical and Laboratory Standards Institute guidelines; it is considered a fastidious bacterium requiring blood or blood products, and modified atmospheric conditions such as 5% CO_2 (CLSI Guideline M45-A2) (235). For this work, MICs were determined in the

same TSB medium used for biofilm assays, allowing direct comparison to biofilm data. Briefly, β-lactams were serially diluted two-fold in DMSO and incubated with overnight cultures of *L. monocytogenes* that were standardized to an OD_{600} ~0.05 in TSB (1 μL of β-lactam solution plus 99 μL culture, total 100 μl with a final concentration of 1% DMSO v/v) at 37°C. Growth of controls was unaffected by the addition of 1% DMSO (236). Growth was monitored at 24 h and 48 h, and the MIC defined as the lowest concentration resulting in no growth after 48 h compared to control samples.

L. monocytogenes biofilm formation and dispersal assays

Biofilm assays were performed as previously described (59). Briefly, in the biofilm formation assay, overnight cultures of *L. monocytogenes* were diluted to an OD_{600} ~0.03 in TSB. β-lactams at the indicated concentrations were added to each well (1.5 μL) and incubated with the *L. monocytogenes* culture (148.5 μL). The biofilms were grown on polystyrene pegs for 72 h (3 passages x 24 h each). In the dispersal assay, biofilms were grown in TSB plus 1% DMSO (v/v) for 72 h (3 passages x 24 h each) and then washed with 1X phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 at pH 7.4) for 10 min. Following the wash step, the biofilms on the pegs were transferred to a 96-well plate that contained 148.5 μL TSB plus 1.5

μL β-lactams in DMSO (at the indicated concentrations) or DMSO alone (vehicle control) in each well and then incubated at 37^{o}C, 24 h, 200 rpm. The amount of biofilm that formed (or remained) on the pegs was quantified using 0.1% w/v crystal violet (CV) and the absorbance of the eluted CV was read at 600 nm using a plate reader as previously described (59). The planktonic cell optical density and CV absorbance graphs were generated using GraphPad Prism 5 (GraphPad Software, Inc.). Statistical values (*P*-values) were calculated using the one-way analysis of variance (ANOVA) test and Dunnett's post test software packages in GraphPad Prism 5.

Construction of *ΔcesK* and Δ*pbpD1* mutants

To generate *L. monocytogenes* EGD-e *cesK* (*lmo2421*) and *pbpD1* (*lmo2754*) deletion constructs, four primers containing restriction sites for subsequent cloning were synthesized for each gene (Supplementary Table S1) to amplify fragments corresponding to the regions 1 kb up and downstream of the gene to be deleted. PCR products were digested with the appropriate restriction enzymes and purified using a gel extraction kit (Fermentas) kit. The purified fragments were ligated into the temperature sensitive pAUL-A vector that was digested with EcoRI and SalI for Δ*cesK* and SacI and SalI for Δ*pbpD1*. The ligation mixtures were introduced into *E. coli* DH5α by electroporation at 2.5 kV (237) using the *E. coli* Pulser

apparatus (BioRad) and transformants were selected on LB agar containing 300 μg/ml erythromycin (Erm).

Electroporation into *L. monocytogenes* EGD-e

L. monocytogenes EGD-e was made electrocompetent following previously published protocols (238, 239) with some modifications. Briefly, a single colony of *L. monocytogenes* EGD-e was grown in 5 mL BHI broth overnight at 37°C, 200 rpm shaking. Following incubation, Penicillin G was added to a final concentration of 10 μg/ml for an additional 2 h and the culture (2 mL) was harvested by centrifugation using the Eppendorf 5415D centrifuge at 16,100x g. The pellet was washed twice with nuclease-free water (Qiagen) and resuspended in 1 mL water and lysozyme (final concentration 100 μg/mL). The cell suspension was incubated in a 37°C water bath for 30 min and then washed and resuspended in 100 μL nuclease-free water. The deletion construct (5 μL of 100 ng/μL DNA) was electroporated into 100μL of *L. monocytogenes* EGD-e using 2 mm electroporation cuvettes (Boca Scientific) and *E. coli* Pulser apparatus (BioRad) set at 2.5 kV. Following electroporation, 5 mL of BHI broth was added and the cells were incubated at 30°C, 4 h. The cells were harvested at 16,100x g for 1 min, resuspended in 100 μL BHI, spotted onto BHI agar and incubated overnight at 30°C. After incubation, the cells were scraped off the plate using calibrated disposable inoculating loops (BD

Biosciences) and resuspended in 1 mL BHI broth. The cell suspension (100 μL) was plated on BHI agar containing Erm (5 μg/mL) and incubated at 30°C for 3 d. Single colonies were streaked on a fresh BHI-Erm5 agar plate and incubated at 37°C overnight to select for merodiploids. Merodiploids were streaked onto BHI agar and incubated at 42°C for 2 d. To identify deletion mutants, single colonies were streaked in parallel on BHI and BHI-Erm 5 agar and incubated at 42°C for 2 d. Erm-sensitive colonies were analyzed by colony PCR with their respective deletion primers (Table S3.1) to confirm deletion of the gene of interest.

PBP profiling using Bocillin-FL

To identify the PBPs targeted by the β-lactams of interest, a competitive Bocillin-FL (Boc-FL) assay (141) was used, with modifications. A 5-mL overnight culture was standardized to OD_{600} ~ 0.03 in fresh TSB with or without drug (4950 μL standardized culture plus 50 μL of β-lactam solution at the indicated concentrations) and incubated overnight at 37°C, 200 rpm. Following incubation, the cells were harvested by centrifugation using the Beckman Coulter® Allegra X-12 centrifuge at 2095 x g for 10 min, and the pellet resuspended in 250 μL 1X PBS. The cells were lysed using a FastPrep®-24 Instrument (MPBio) at 6.0 m/s for 1 min x 2, with a 5 min incubation on ice between treatments. The supernatants were collected via centrifugation at 21,130x g using the Eppendorf 5424 R

centrifuge for 1 min at 4°C. The supernatants (45 μL) were incubated with ethylenediaminetetraacetic acid (final concentration of 10 mM) for 30 min at 37°C to prevent degradation of Boc-FL (Invitrogen) by β-lactamases (141). Boc-FL was added to each sample (final concentration 0.5 μM), and then incubated for an additional 30 min at 37°C. This concentration of Boc-FL (0.5 μM) was chosen for the assay because PBPs could not be visualized at concentrations <0.05 μM, and concentrations >0.5 μM resulted in significant non-specific fluorescence. The samples were separated on 10% SDS-PAGE gels for ~ 5.5 h, 80V and imaged using a Typhoon Trio+ Variable Imager with excitation wavelengths of 532 or 633 nm (Boc-FL and protein mass standards, respectively) and emission filters 526BP and 670BP30.

Results

Identification of small molecules that inhibit *L. monocytogenes* biofilm formation and/or disperse established biofilms

The Previously-Approved Drugs (PAD) library (234) was screened at an initial concentration of 10 μM and molecules capable of modulating *L. monocytogenes* biofilm formation and dispersal were identified. The pre-biofilm assay – where biofilms were grown in the presence of compounds for 3 d – yielded 147 (13.1%) planktonic growth and/or biofilm inhibitors (defined as ≤50% of vehicle-treated control), or biofilm

stimulators (≥200% compared to the vehicle-treated control; Supplementary Table S3.2). In the biofilm dispersal assay, 69 hits (6.1%) were identified as dispersants (<50% biofilm remaining compared to vehicle-treated control), growth inhibitors of cells shed from the biofilm, growth stimulators, or biofilm stimulators (Supplementary Table S3.3). The molecules fall into several classes, including antibacterials, antifungals, vitamins as well as several with activities on human cells. In total, 37 drugs, of which 33 were antibiotics, both inhibited biofilm formation and dispersed established biofilms. Of the antibiotics, 23 were β-lactams, while the rest were fluoroquinolones, quaternary ammonium compounds, biguanides, oligopeptides or glycopeptides. We focused here on β-lactams, as the majority of the antibiotic hits (>48%) were of this class, and – despite their well-established mechanism of action – had some unexpected effects on *L. monocytogenes* biofilm formation.

To establish whether the molecules of interest were active on other strains of *L. monocytogenes*, the minimal inhibitory concentrations (MICs) of the compounds of interest for 4 strains were determined (Supplementary Table S3.4). *L. monocytogenes* 568, EGD-e and food isolates belonging to serotypes 1/2a and 1/2b have similar MICs (within 1 2-fold dilution), ranging from 0.16 – 10 μM. *L. monocytogenes* is less susceptible to most cephalosporins as compared to the penicillins (138). With the exception of cefalonium and cefotiam (MICs of 1.6 and 3.1 μM,

respectively), the MICs of the cephalosporins were all ≥50 μM. Some had no effect on growth even at the highest concentration tested (500 μM; data not shown). As it was insoluble at higher concentrations, the highest concentration of cefaclor tested was 10 μM.

Stimulation of *L. monocytogenes* biofilm formation by sub-MIC β-lactams

Since the 10 μM concentration used for the initial screen was above the MIC for many of the β-lactams listed in Table S3.4, their effects on biofilm development were further tested in a dose-response assay (Supplementary Figure S3.1). For ampicillin (MIC of 0.31 μM), concentrations near the MIC (0.16 μM) decreased both planktonic cell density (~75% of control) and biofilm formation (~24% of control). However, at concentrations between 0.02-0.08 μM, there was a small decrease in planktonic cell density (to ~70% of control), but biofilm formation was unexpectedly increased, to a maximum of >280% of control (Figure 3.1a). Similarly, other penicillin derivatives simulated biofilm formation at sub-MIC concentrations (Figure S3.1), although the concentrations at which biofilm stimulation occurred varied. The ureidopenicillin piperacillin did not stimulate biofilm formation to the same extent under these conditions, although the increase was statistically

significant. At the lowest concentration tested (0.04 μM), biofilm formation was ~130% of control (Figure S3.1).

In addition to the penicillins, select cephalosporins stimulated biofilm formation at sub-MIC levels (Figure S3.1). In the biofilm inhibitory assay, planktonic cell density was not significantly altered in the presence of cefotaxime (0.04 – 1.25 μM), but there was a marked decrease in biofilm formation (~20% compared to control) at 1.25 μM (Figure 3.1b). At concentrations <0.16 μM, planktonic cell density was unaffected, and biofilm formation was stimulated (>200% compared to control). Biofilm formation was also stimulated by sub-MIC cefalonium, cefixime, cefotiam, and cefuroxime (Figure S3.1).

Sub-MIC cefoxitin has weak biofilm stimulatory effects, but sub-MIC cefmetazole does not stimulate biofilm formation

Cefoxitin had a weak, but statistically significant, biofilm stimulatory effect (~130% compared to control, *** $P< 0.001$) compared with the >200% stimulation seen with similar concentrations of cefotaxime (Figure 3.1b), and biofilm formation decreased with increasing concentrations (Figure 3.1c). In contrast, cefmetazole did not stimulate biofilm formation (Figure 3.1d). At 0.04 μM, biofilm formation was ~110% of control (not statistically significant), and at higher concentrations, there was an inverse correlation between concentration and biofilm. Cefaclor had no effect on

biofilm formation, but the concentration was restricted to 10 μM or less, due to its limited solubility (Figure S3.1).

β-lactams disperse established *L. monocytogenes* biofilms

Specific β-lactams dispersed established biofilms and/or inhibited the growth of cells shed into the planktonic phase (Supplementary Figure S3.2). For example, ampicillin at concentrations above the MIC (0.31 μM) inhibited the growth of shed planktonic cells (Figure 3.2a) to ~10% of control, but the level of inhibition did not improve with increasing ampicillin concentrations (0.63 – 20 μM; Figure 3.2a). Similarly, the amount of biofilm remaining was concentration-independent, ~20% of control over the range tested. Treatment of established biofilms with other penicillin derivatives in the same concentration range (0.63 – 20 μM) also caused dispersal (Figure S3.2). However, beyond a threshold, the amount of biofilm remaining did not further decrease. With increasing drug concentrations to 20 μM (representing 4 to 64 times the MIC, depending on the drug) ~20-25% of control biofilm remained (Figure S3.2).

When established biofilms were exposed to cephalosporins, all caused dispersal except for cefaclor, which had no effect at the concentrations tested (Figures 3.2b-d and S3.2). Many dispersed established biofilms up to a certain concentration, beyond which there was no further dispersal (Figure S3.2). For example, cefotaxime (Figure 3.2b)

had effects similar to ampicillin (Figure 3.2a) despite having a much higher MIC of >100 μM (versus 0.31 μM for ampicillin). At concentrations >2.5 μM, the amount of biofilm remaining on the pegs (~20%) was concentration-independent, with a decrease in planktonic cell density at higher concentrations. For other cephalosporins, the amount of biofilm remaining was inversely correlated with concentration. Cefoxitin and cefmetazole are examples of cephalosporins that dispersed established biofilms in a concentration-dependent manner (Figures 3.2c and 3.2d). At the highest concentration tested (20 μM), the amount of biofilm remaining was ~20% and 10% of control, respectively. In no case were biofilms completely dispersed (Figure S3.2).

Biofilm stimulation is not dependent on the two-component system CesRK

L. monocytogenes has 2 different two-component systems (TCS) – LisRK and CesRK – that are activated by cephalosporins (172). We hypothesized that biofilm stimulation may be among the responses of CesRK to sub-MIC cephalosporins. To test this hypothesis, a Δ*cesK* mutant was generated and compared to the wild type in a biofilm assay at a sub-MIC range of the stimulatory drug, cefotaxime. As shown in Figure 3.3, the Δ*cesK* mutant formed comparable levels of biofilm to the wild type. In the presence of 0.04-0.08 μM cefotaxime, biofilm formation for

Δ*cesK* was comparable to wild type. With increasing concentrations of cefotaxime (>0.31 μM), there was a similar reduction in biofilm formation for both.

Inhibition of PBPD1 impairs biofilm stimulation

To gain insight into why only a subset of β-lactams stimulated *L. monocytogenes* biofilm development, we looked at the target specificities of the drugs of interest. A previous study reported that 8 out of 10 putative PBPs encoded by *L. monocytogenes* could be detected using high concentrations (50 μM) of fluorescent β-lactam derivatives and that a ninth PBP (PBPD2), was detectable, but only when overexpressed in *E. coli* (141). However, only 5 PBPs were previously identified using 5 μg/ml of radiolabelled penicillin: PBPs 1 through 5 (138, 139), since renamed PBPA1, PBPB2, PBPB1, PBPA2 and PBPD1 (141). We first used a Boc-FL assay to identity the *L. monocytogenes* PBPs expressed under our experimental conditions. In control samples, 0.5 μM Boc-FL labeled 6 PBPs: PBPA1, PBPB2, PBPB1, PBPA2, PBPB3 and PBPD1 (Figure 3.4). Higher concentrations of Boc-FL did not increase the number of PBPs detected, and caused unacceptable levels of non-specific labeling (data not shown).

To determine which PBPs were inhibited by the β-lactams of interest, a competition assay was performed. PBPs that are covalently

modified by a particular β-lactam can no longer be labeled by Boc-FL, and thus do not appear on a fluorescent PBP profile. The stimulatory cephalosporin, cefotaxime, bound to PBPA1, PBPB2 and PBPA2 at the highest concentrations tested in the biofilm inhibitory assay (Figure 3.4a). At concentrations that stimulated biofilm formation (0.04 – 0.16 μM), PBPB2 and PBPA2 were not detected in cefotaxime-treated cells (Figure 3.4a).

At higher concentrations, the weak stimulator cefoxitin and the non-stimulator cefmetazole also bound to PBPA1, PBPB2 and PBPA2 (Figure 3.4b and c, respectively). At lower concentrations, both drugs bound PBPB2 and PBPA2, while PBPA1 fluorescence was reduced compared to control. LMW PBPD1 was bound by cefmetazole at all concentrations tested (Figure 3.4c) and by higher concentrations of cefoxitin (Figure 3.4b). At low concentrations of the weak stimulator cefoxitin (0.04 – 0.31 μM), PBPD1 fluorescence was reduced compared with the untreated control.

Biofilm stimulation is attenuated in the Δ*pbpD1* mutant

Because PBPA1, PBPB2 and PBPA2 were bound by all cephalosporins tested, but stimulatory drugs specifically did not inactivate PBPD1, we hypothesized that PBPD1 activity was required for biofilm stimulation in response to β-lactam treatment. A Δ*pbpD1* mutant was

generated and its biofilm phenotype in response to stimulatory, weakly stimulatory, and non-stimulatory cephalosporins tested as described above. Compared to the parental strain, the Δ*pbpD1* mutant's response to stimulatory drugs was attenuated (Figure 3.5). With 0.04 μM cefotaxime, wild-type biofilm formation was ~380% of control whereas that of Δ*pbpD1* was ~140%, while biofilm formation was ~230% for the parental strain but below control levels for the Δ*pbpD1* mutant with 0.16 μM cefotaxime. At concentrations >0.31 μM, biofilm formation was reduced for both wild type and Δ*pbpD1* strains (Figure 3.5).

Discussion

In our screen of 1120 previously FDA-approved, off-patent drugs, we identified 147 (13.1%) that decreased *L. monocytogenes* biofilm formation and 69 (6.1%) that dispersed established biofilms. These hit rates were relatively high due to the nature of the library, which is composed entirely of known bioactives (32). We focused here on the β-lactams due to their well-characterized target profiles but unexpected effects on *L. monocytogenes* biofilms.

The PBP targets of β-lactams have been implicated in biofilm formation in a few mutant studies (136, 145, 240), though in most cases, specific mechanisms were unclear. In *Streptococcus gordonii*, inactivation of genes coding for PBPB2 and PBP5 impaired biofilm development (240).

In *E. coli*, deletion of a HMW PBP, PBP1b, reduced swimming motility and biofilm formation (241) and deletion of LMW PBPs (PBP4, 5, 7) alone or in combination decreased biofilm formation, with the triple mutant having the largest reduction (136). A transposon mutagenesis screen of *L. monocytogenes* 10403S identified a number of cell envelope biosynthesis genes required for biofilm formation, including the gene encoding PBPA2, previously implicated in β-lactam resistance (48, 143). Although other PBPs in *L. monocytogenes* have been investigated for their roles in β-lactam resistance, growth and morphology (48, 138, 139, 141-144, 242), no links with biofilm development have been established.

The biofilm-related effects of β-lactams identified in this work were in many cases independent of bacterial killing. For example, many cephalosporins are considered ineffective against *L. monocytogenes* due to high MICs (Table S3.4), but they clearly modulate biofilm formation and dispersal at concentrations well below the MIC (Figures 3.1, S3.1 and S3.2). Even β-lactams to which *L. monocytogenes* is susceptible can stimulate or disperse biofilms at sub-MIC levels. Sub-MIC antibiotics – including β-lactams – have previously been reported to impair biofilm formation, although in most cases, the exact mechanism was not determined. Adhesion of *E. coli* to catheters was decreased by sub-MIC cefotaxime (243), and sub-MIC cefazolin inhibited the later stages of *Staphylococcus epidermidis* biofilm formation (244).

There are also reports of biofilm stimulation upon exposure to sub-MIC antibiotics (103, 215, 245, 246), similar to the phenotype observed this study (Figures 3.1a-b and S3.1). The biofilm stimulation phenotype, which we propose is a defensive response of *L. monocytogenes* to low concentrations of a subset of cell wall-active antibiotics, was dependent upon the activity of PBPD1, a D,D-carboxypeptidase (144). The ability of a drug to stimulate biofilm formation was inversely correlated with its ability to specifically inactivate PBPD1 (Figure 3.4 and Supplementary Figure S3.3), and Δ*pbpD1* biofilm formation upon exposure to stimulatory β-lactams was markedly attenuated compared to wild type (Figure 3.5). The biofilm stimulatory response to β-lactams requires PBPD1 activity in the context of inactivated HMW PBPs, as loss of PBPD1 alone was not sufficient to increase biofilm formation in the absence of drug (Figure 3.5, vehicle control lane).

How might PBPD1 activity in the absence of HMW PBP activity lead to biofilm stimulation? Many D,D-carboxypeptidases are also endopeptidases; an example is *E. coli* AmpH (247). PBPD1 belongs to the AmpH family (144), but only its D,D-carboxypeptidase activity has been formally tested. If PBPD1 has endopeptidase activity, an increase in the amount of muropeptide fragments released by cleavage of stem peptides in the absence of new PG synthesis could act as a signal to induce biofilm formation, as demonstrated for Gram negative bacteria (102, 248, 249).

In many bacteria, increased EPS synthesis has been linked to β-lactam exposure. Sub-inhibitory concentrations of imipenem induced expression of both AmpC β-lactamase and the EPS polysaccharide, alginate, in *P. aeruginosa* (102). Increased concentration of cytoplasmic anhydromuropeptides due to perturbation of cell wall turnover by imipenem leads to binding of the transcriptional activator AmpR, resulting in AmpC expression (248). Similarly, an increase in alginate production in an *ampDE* double mutant of *Azotobacter vinelandii* impaired in peptidoglycan recycling was proposed to result from increased levels of cytoplasmic anhydromuropeptides (249). In *E. coli*, select β-lactams (carbenicillin, cefotetan, cephaloridine, cephalothin, and ticaricillin) induced the expression of colanic acid, an exopolysaccharide required for biofilm formation and maintenance of biofilm architecture (106, 250). Increased colanic acid production was not due to stresses that accompanied cell death nor general inhibition of PG synthesis, because (as seen in this work) only a subset of β-lactams – and not DNA replication or protein synthesis inhibitors – increased colanic acid expression (106). In *S. epidermidis*, sub-MIC antibiotics (e.g. tetracycline, tigecycline, and quinupristin-dalfopristin) potentially induced the expression of the *ica* operon which encodes for poly-*N*-acetylglucosamine (PNAG), a major component of the EPS (103, 105, 251).

Because *Listeria* lacks genes encoding enzymes for polysaccharide production (4, 46), *L. monocytogenes* biofilm stimulation by sub-MIC β-lactams could result from increased levels of extracellular DNA (eDNA). Along these lines, stimulation of biofilm formation in some *S. aureus* strains by sub-MIC methicillin requires expression of the AtlA autolysin, a PG hydrolase, which enhances release of eDNA (104). In addition to eDNA, *L. monocytogenes* EPS contains proteins (37). Protease pretreatment of *L. monocytogenes* reduced the levels of the surface-exposed Ami4b autolysin, resulting in decreased biofilm formation (47). We showed recently that the EPS-degrading enzymes DNase and proteinase K disperse ~75-100% of biofilms grown under both normal and sub-MIC ampicillin-induced stimulated conditions, suggesting that the EPS of stimulated biofilms contains the same components as unstimulated biofilms (252). In addition, when biofilms were grown in the presence of sub-MIC ampicillin and DNase or proteinase K, biofilm stimulation was abolished (Supplementary Figure S3.4).

In addition to stimulating biofilm formation, sub-MIC β-lactams dispersed established *L. monocytogenes* biofilms (Figure S3.2), an interesting finding as biofilms are typically considered more tolerant of antibiotics than planktonic cells (44, 45). Small molecules or fatty acids can cause dispersal of biofilms (76, 207, 226, 253), as can degradation of the EPS matrix (37, 46, 254). In our assays, none of the β-lactams tested

could fully disperse established biofilms (Figures 3.2 and S3.2) nor completely inhibit growth of shed cells. This pattern matches the biphasic killing reported for persister cells, where the bulk of the population is killed at high doses, but beyond a certain threshold, increasing concentrations of drug do not kill the remaining cells (plateau effect) (117, 255). The inability of β-lactams to disperse dormant persister cells implies that dispersal requires cell wall turnover.

Many cephalosporins dispersed a substantial amount of established *L. monocytogenes* biofilm at concentrations well below the MIC (Figures 3.2b-d and S3.2). For example, Figure 3.2b shows that ~65% of established biofilm was removed by treatment with 0.63 μM cefotaxime, over 100-fold below the MIC (>100 μM). Although there is no apparent effect on growth, several HMW PBPs are inhibited at this concentration (Figure 3.4a). We speculate that inhibition of HMW PBP activity by β-lactams could affect the display of sortase-dependent adhesins (121, 130) on the cell surface, as they must be incorporated into the cell wall during PG synthesis. Loss of surface adhesins would not be expected to impact growth, but could decrease biofilm integrity.

Inhibition of PG synthesis by β-lactams triggers specific cell wall stress responses. Four TCSs have been reported to respond to cell wall stress in *L. monocytogenes:* VirRS, LiaSR, LisRK, and CesRK (171-174, 181). LisRK and CesRK are activated by ethanol and β-lactams in

planktonic cells (171-173, 181, 182) and positively regulate genes encoding PBPA2 and PBPD2, a putative transporter that may have a role in β-lactam resistance, and a LytR-like regulatory protein (171, 172, 182). Genes highly induced in planktonic cells by cefuroxime – a cephalosporin that stimulated biofilm formation at sub-MIC levels in our assay (Figure S1) – are LisR/CesR-dependent (173). However, the wild type and Δ*cesK* mutant responded similarly to both sub-MIC cefotaxime (Figure 3) and ampicillin (data not shown), suggesting that CesRK is not involved in the biofilm stimulation response. Comparative transcriptome analysis of *L. monocytogenes* biofilm versus planktonic cells revealed that *liaS* and *cesK* are down-regulated in biofilm cells, while *virS* or *lisK* expression was unchanged (112), suggesting they are not involved in biofilm formation, and supporting the lack of a biofilm phenotype for the Δ*cesK* mutant. In addition, none of the genes regulated by LiaSR have been reported to function in biofilm formation (173, 177).

In conclusion, our screen identified a variety of bioactive small molecules that modulate *L. monocytogenes* biofilm formation. The proposed mechanisms of actions of these compounds vary, and they elicit a range of biofilm responses (inhibition, stimulation, and/or dispersal). The ability to provoke biofilm responses at concentrations that have no apparent effects on bacterial growth suggests that biofilm modulation is a sensitive phenotype for assessing potential effects on bacterial physiology.

A subset of the hits, β-lactams, had effects ranging from biofilm stimulation, inhibition and/or dispersal that were dependent on drug structure and dose. Determining how ostensibly 'ineffective' β-lactams such as cephalosporins disperse established biofilms, even at concentrations well below the MIC, will shed light on the process of *L. monocytogenes* biofilm development and improve our understanding of antibiotic action.

Acknowledgements

We thank Lisbeth Truelstrup-Hansen for providing strains and plasmids. This work was supported by in part by a Tier I Canada Research Chair to G.D.W., a Natural Sciences and Engineering Research Council (NSERC) Undergraduate Summer Research Award to A.A., and funding provided by the Canadian Food Inspection Agency (CFIA). Notwithstanding, the CFIA does not necessarily endorse the views or practices presented by the authors in this manuscript.

Figure 3.1

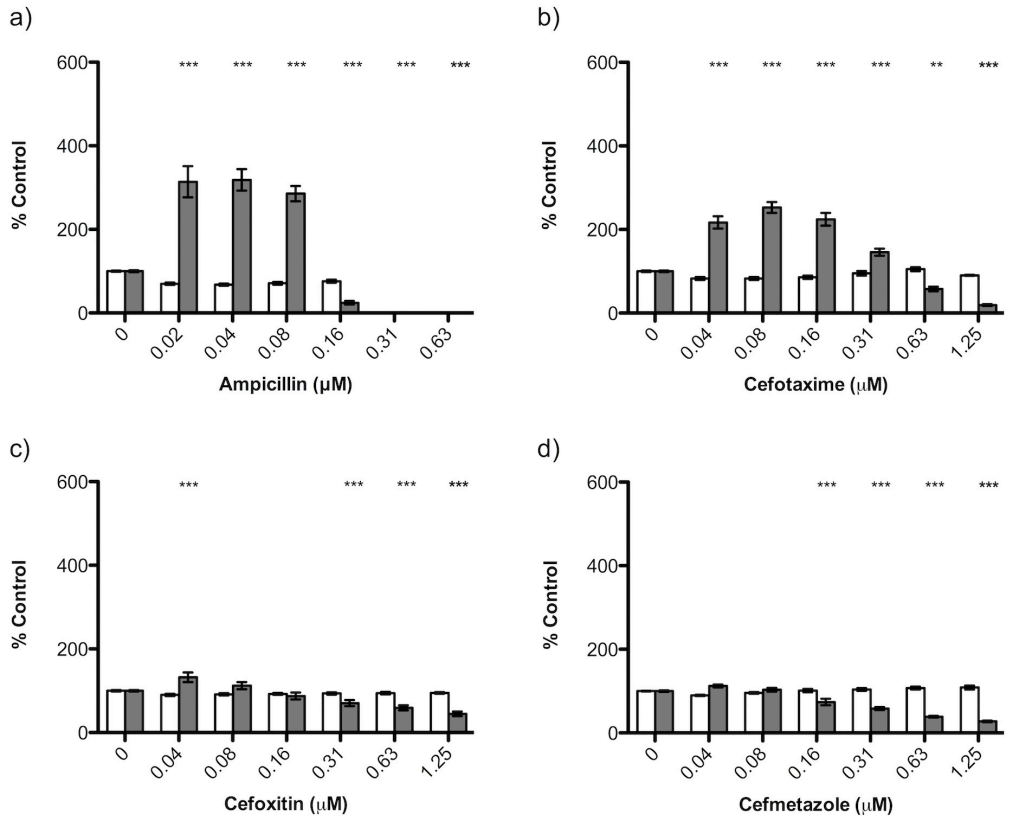
a)
b)
c)
d)
% Control
600
400
200
0
0
0.02
0.04
0.08
0.16
0.31
0.63
1.25
Ampicillin (μM)
Cefotaxime (μM)
Cefoxitin (μM)
Cefmetazole (μM)
***
**

Figure 3.1. Select sub-MIC β-lactams stimulate biofilm formation. *L. monocytogenes* biofilms were grown with (a) ampicillin, (b) cefotaxime, (c) cefoxitin, or (d) cefmetazole at the concentrations indicated, and biofilm formation was quantified using crystal violet staining. Both sub-MIC ampicillin and sub-MIC cefotaxime stimulated biofilm formation (>200%). Planktonic cell density (white bars) and biofilm formation (grey bars) are expressed as a percentage of control. n≥3, with standard error shown. ** $P<0.01$; *** $P< 0.001$

Figure 3.2

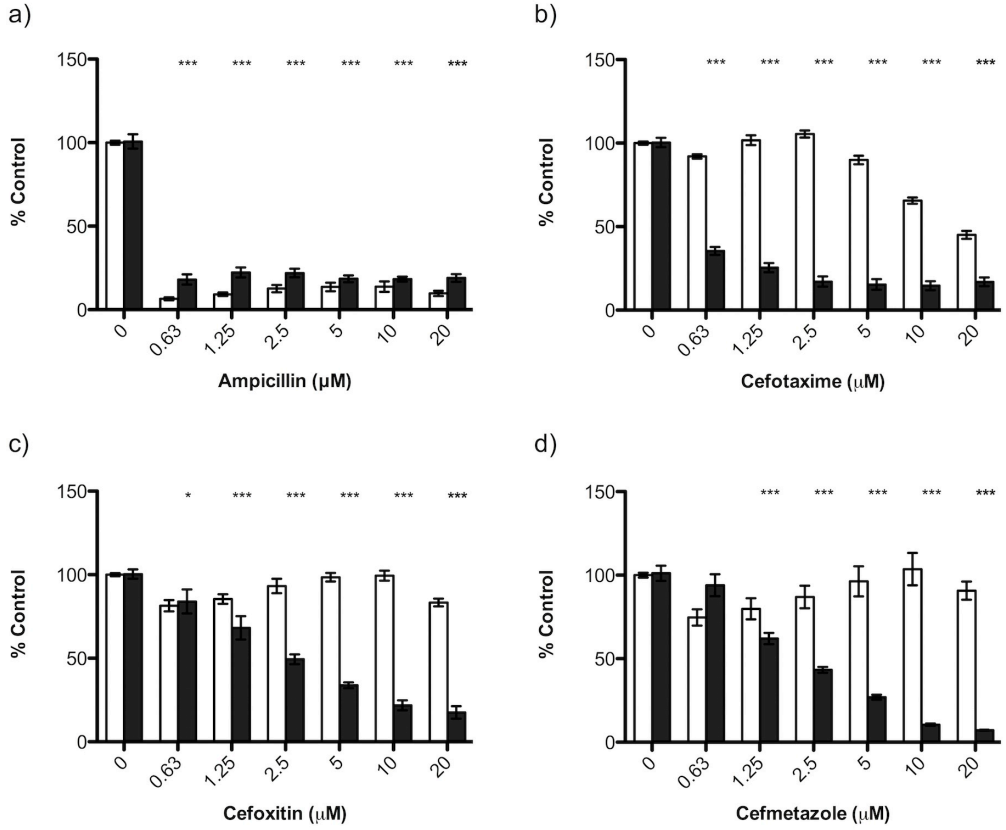
a)
b)
c)
d)
% Control
150
100
50
0
0
0.63
1.25
2.5
5
10
20
Ampicillin (μM)
Cefotaxime (μM)
Cefoxitin (μM)
Cefmetazole (μM)
*
***

Figure 3.2. β-lactams disperse established biofilms. Established *L. monocytogenes* 568 biofilms were treated with (a) ampicillin, (b) cefotaxime, (c) cefoxitin, or (d) cefmetazole at the concentrations indicated, and quantified using crystal violet staining. All β-lactams dispersed established biofilms but efficacy depended on concentration. Density of planktonic cells shed from the biofilms (white bars) and biofilm remaining on the peg after treatment (black bars) are expressed as a percentage of control. n≥3, with standard error shown.

* $P<0.05$; *** $P< 0.001$

Figure 3.3

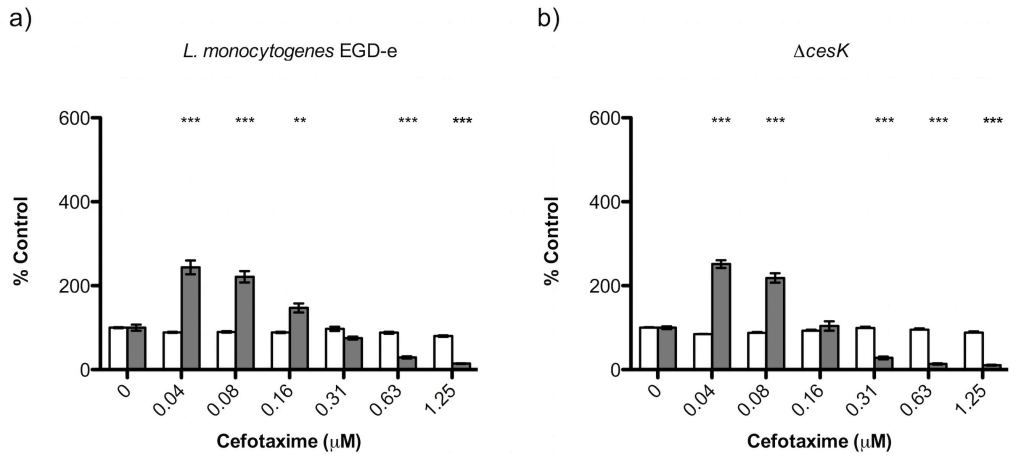
a)
L. monocytogenes EGD-e
b)
ΔcesK
% Control
600
400
200
0
***
**
0
0.04
0.08
0.16
0.31
0.63
1.25
Cefotaxime (μM)

Figure 3.3. The biofilm response of a Δ*cesK* mutant to stimulatory β-lactams is similar to wild type. Biofilms of (a) the parental strain, *L. monocytogenes* EGD-e and (b) an isogenic Δ*cesK* mutant were grown with cefotaxime at the concentrations indicated. Planktonic cell density (white bars) and biofilm formation (grey bars) are expressed as a percentage of control. n=3, with standard error shown.

** $P<0.01$; *** $P<0.001$

Figure 3.4

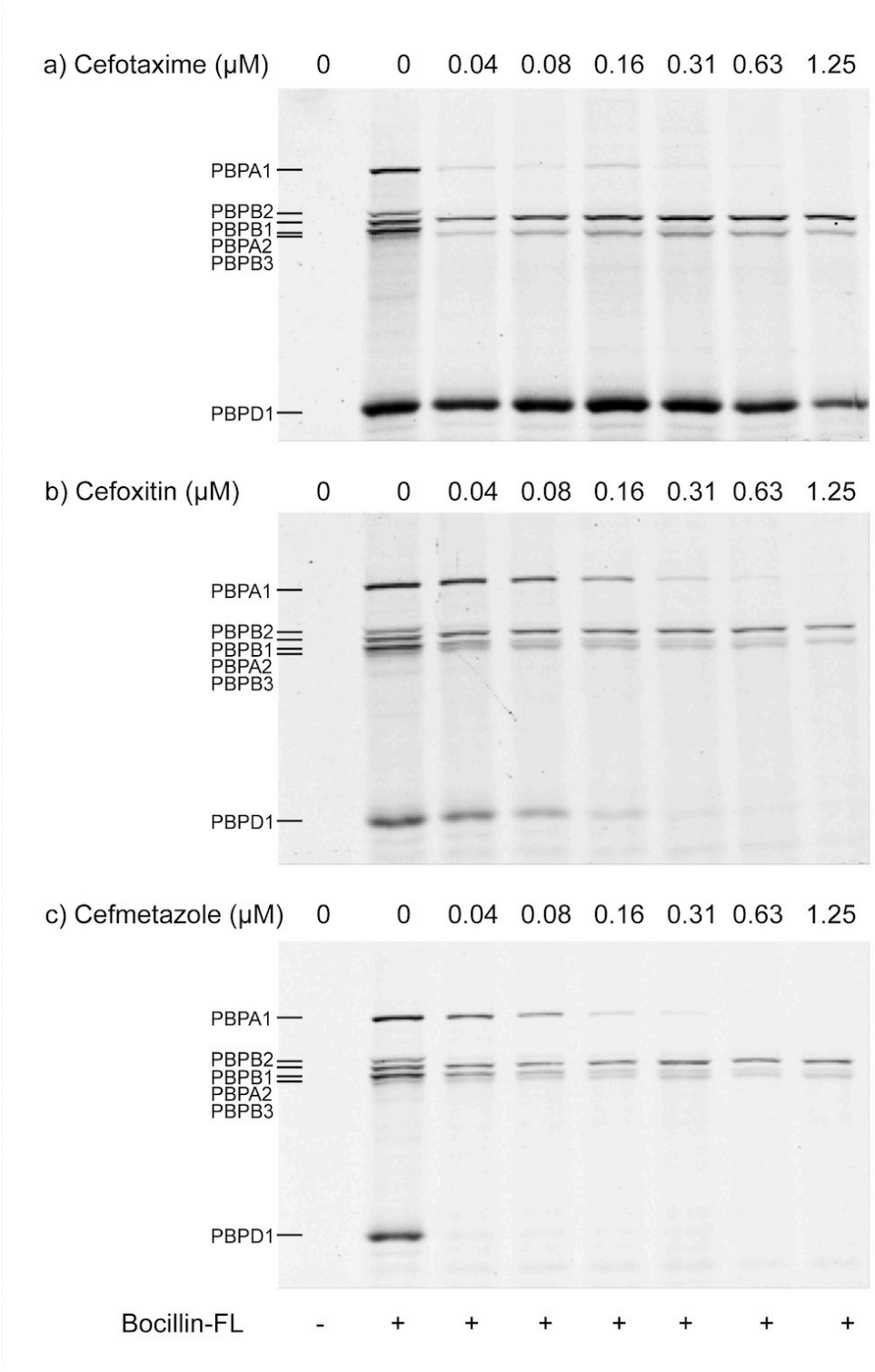
a) Cefotaxime (μM) 0 0 0.04 0.08 0.16 0.31 0.63 1.25
PBPA1
PBPB2
PBPB1
PBPA2
PBPB3
PBPD1
b) Cefoxitin (μM) 0 0 0.04 0.08 0.16 0.31 0.63 1.25
PBPA1
PBPB2
PBPB1
PBPA2
PBPB3
PBPD1
c) Cefmetazole (μM) 0 0 0.04 0.08 0.16 0.31 0.63 1.25
PBPA1
PBPB2
PBPB1
PBPA2
PBPB3
PBPD1
Bocillin-FL - + + + + + + +

Figure 3.4. Penicillin-binding-protein profiles of β-lactam-treated *L. monocytogenes*. *L. monocytogenes* was grown with (a) cefotaxime, (b) cefoxitin or (c) cefmetazole at the concentrations indicated, and whole cell lysates labelled with Bocillin-FL (final concentration 0.5 μM) as described in the Methods. All cephalosporins targeted PBPA1, PBPB2, PBPA2, while PBPD1 was targeted only by non- or weakly-stimulatory cephalosporins.

Figure 3.5

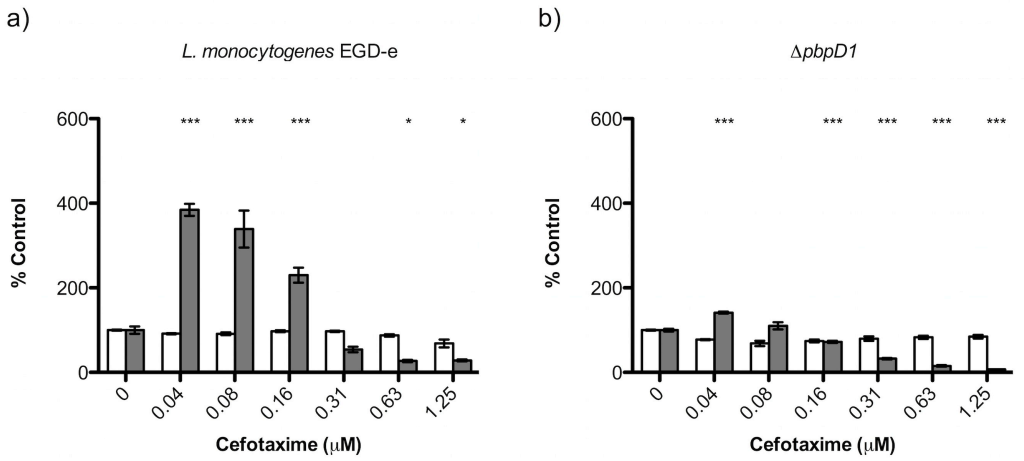
a)
L. monocytogenes EGD-e
% Control
600
400
200
0
***
***
***
*
*
0
0.04
0.08
0.16
0.31
0.63
1.25
Cefotaxime (μM)
b)
ΔpbpD1
% Control
600
400
200
0
***
***
***
***
***
0
0.04
0.08
0.16
0.31
0.63
1.25
Cefotaxime (μM)

Figure 3.5. Biofilm stimulation in response to β-lactams is attenuated in a *pbpD1* mutant. Biofilms of (a) *L. monocytogenes* EGD-e or (b) the Δ*pbpD1* mutant were grown with cefotaxime at the concentrations indicated. The stimulatory effect of cefotaxime on biofilm formation was attenuated for the Δ*pbpD1* mutant compared to the parent strain. Planktonic cell density (white bars) and biofilm formation (grey bars) are expressed as a percentage of control. n=3, with standard error shown. * $P<0.05$; *** $P<0.001$

Figure S3.1

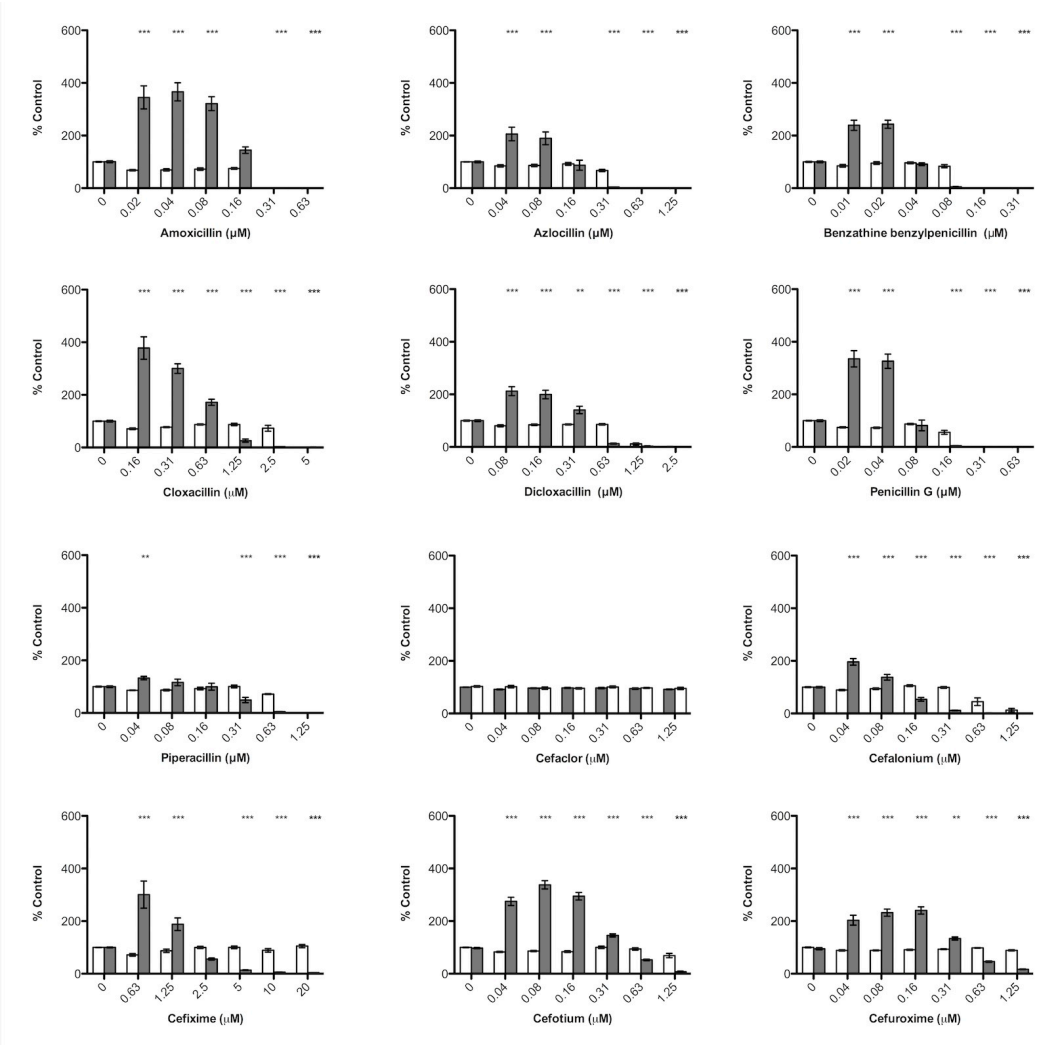
% Control
Amoxicillin (μM)
Azlocillin (μM)
Benzathine benzylpenicillin (μM)
Cloxacillin (μM)
Dicloxacillin (μM)
Penicillin G (μM)
Piperacillin (μM)
Cefaclor (μM)
Cefalonium (μM)
Cefixime (μM)
Cefotium (μM)
Cefuroxime (μM)

Figure S3.1. The effects of β-lactams on biofilm formation. *L. monocytogenes* 568 biofilms were grown on polystyrene pegs and quantified using crystal violet staining. Planktonic growth at day 3 (white bars) and biofilm formation (grey bars), expressed as a percentage of control samples, n≥3. * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Figure S3.2

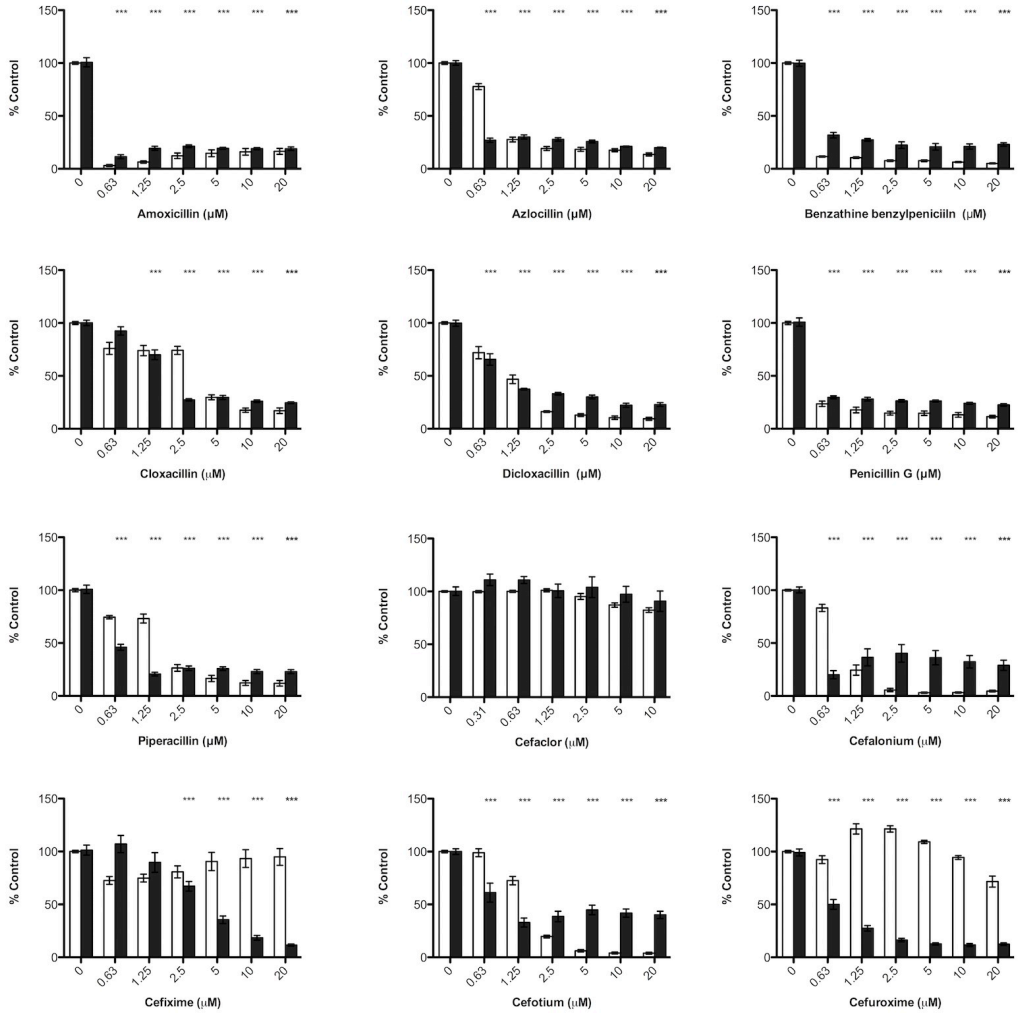
% Control
Amoxicillin (μM)
Azlocillin (μM)
Benzathine benzylpeniciiln (μM)
Cloxacillin (μM)
Dicloxacillin (μM)
Penicillin G (μM)
Piperacillin (μM)
Cefaclor (μM)
Cefalonium (μM)
Cefixime (μM)
Cefotium (μM)
Cefuroxime (μM)

Figure S3.2. The effects of β-lactams on biofilm dispersal. Pre-existing *L. monocytogenes* 568 biofilms were treated β-lactams and quantified using crystal violet staining. Planktonic growth (white bars) and biofilm remaining on the pegs after treatment (black bars), expressed as a percentage of control samples, n≥3. * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Figure S3.3

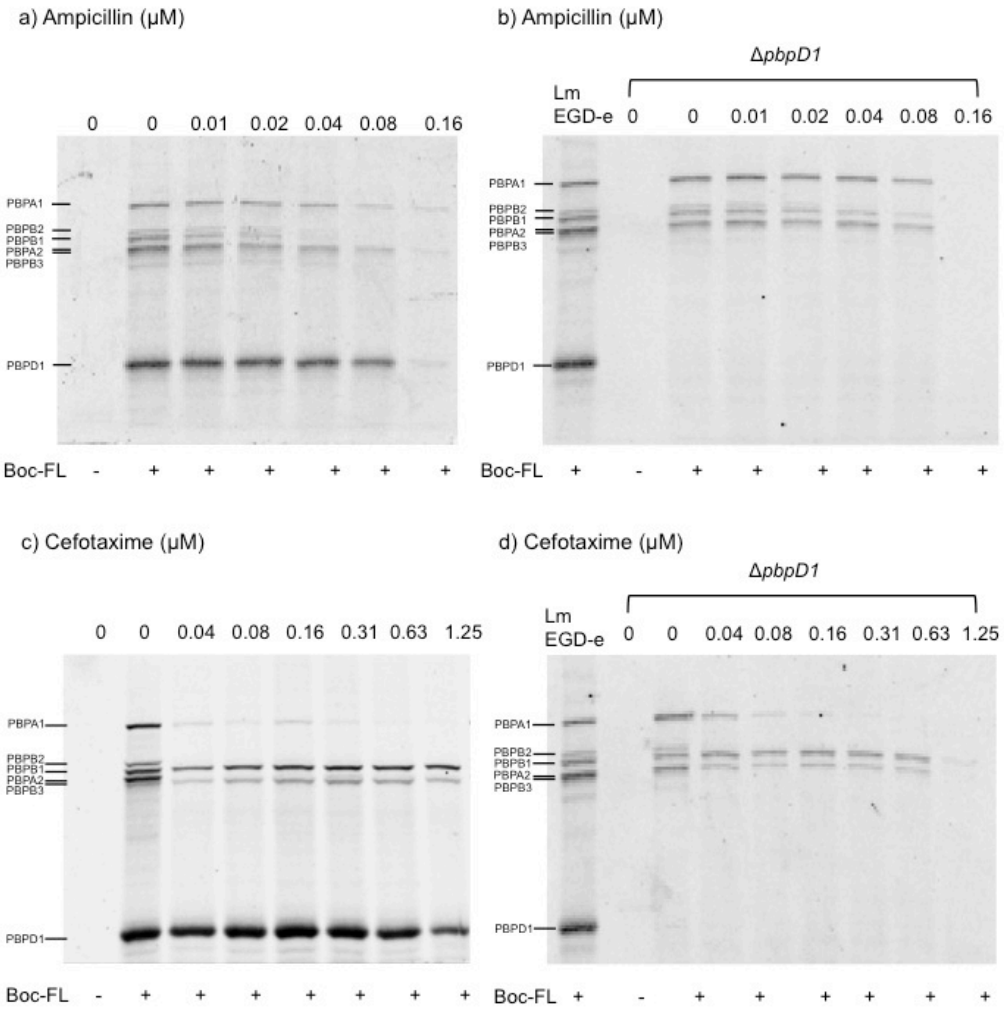
a) Ampicillin (μM)
0 0 0.01 0.02 0.04 0.08 0.16
PBPA1
PBPB2
PBPB1
PBPA2
PBPB3
PBPD1
Boc-FL - + + + + + +
b) Ampicillin (μM)
ΔpbpD1
Lm EGD-e 0 0 0.01 0.02 0.04 0.08 0.16
PBPA1
PBPB2
PBPB1
PBPA2
PBPB3
PBPD1
Boc-FL + - + + + + + +
c) Cefotaxime (μM)
0 0 0.04 0.08 0.16 0.31 0.63 1.25
PBPA1
PBPB2
PBPB1
PBPA2
PBPB3
PBPD1
Boc-FL - + + + + + + +
d) Cefotaxime (μM)
ΔpbpD1
Lm EGD-e 0 0 0.04 0.08 0.16 0.31 0.63 1.25
PBPA1
PBPB2
PBPB1
PBPA2
PBPB3
PBPD1
Boc-FL + - + + + + + +

Figure S3.3. Penicillin-binding-protein profiles of β-lactam-treated *L. monocytogenes* EGD-e and Δ*pbpD1* mutant. *L. monocytogenes* was grown with (a-b) ampicillin or (c-d) cefotaxime at the concentrations indicated and whole cell lysates labelled with Bocillin-FL (final concentration 0.5 μM) as described in the Methods. Ampicillin targeted PBPB2 and PBPB1 at high concentrations in both wild type and Δ*pbpD1* mutant. The same PBPs (PBPA1, PBPB2 and PBPA2) were targeted by cefotaxime in both wild type and Δ*pbpD1* mutant.

Figure S3.4

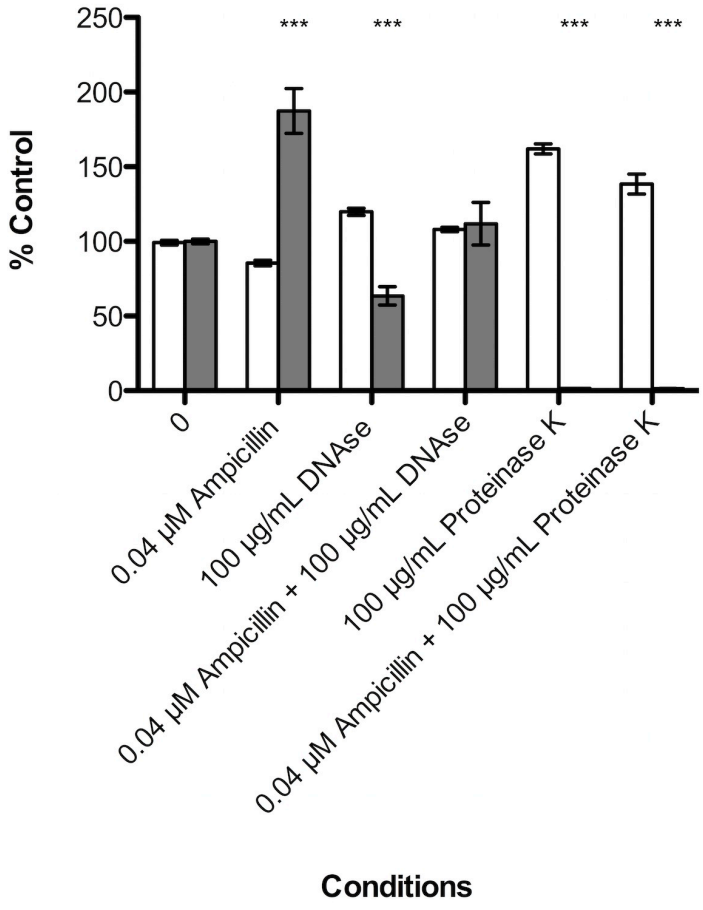
250
200
150
100
50
0
% Control
***
***
***
***
0
0.04 µM Ampicillin
100 µg/mL DNAse
0.04 µM Ampicillin + 100 µg/mL DNAse
100 µg/mL Proteinase K
0.04 µM Ampicillin + 100 µg/mL Proteinase K
Conditions

Figure S3.4. Inhibition of biofilm stimulation by DNase and proteinase K. *L. monocytogenes* 568 biofilms were grown on polystyrene pegs and quantified using crystal violet staining. Planktonic cell density at day 3 (white bars) and biofilm (grey bars) are expressed as a percentage of control, n=2 *** $P<0.001$

Table S3.1. Oligonucleotides used in this study

Primer	Oligonucleotide sequence
cesK EcoRIF1……..	5’-GACGAATTCGAAGAGGCTATCAAGCA-3’
cesK SmaIR1……	5’-TGCCCGGGA AACATTCATCACATGGCTA-3’
cesK SmaIF2…….	5'-ACCCGGGAGTTTTATCGTCAAACTGCCACTA-3'
cesK SalIR2……...	5’-TAGTCG ACCTCTGCTGGATAGGCGT-3'
pbpD1 SacIF1……..	5’-GTTGAGCTCTTGTCGGTCCAACTGGCTC-3’
pbpD1 BamHIR1…..	5’-CAGGATCCATGGGTAATTACGTTTCTGATG-3’
pbpD1 BamHIF2…...	5’-CACTGGATCCAACTTCTCCTTCATTTGAG-3’
pbpD1 SalIR2……	5’-TACGTCGACGTCTCCATGTAGCAGGCT G-3’

Table S3.2. Previously approved drugs that modulate development of *L. monocytogenes* biofilms

Growth inhibitors[a]	Biofilm inhibitors[b]	Biofilm stimulators[c]
Antibacterial	**Analgesic**	**Anticholesteremic**
Florfenicol	Flufenamic acid	Beta-sistosterol
Lasalocid sodium salt	Niflumic acid	**Antihelminthic**
Trimethoprim	Zomepirac sodium salt	Harmine hydrochloride
Aminocoumarins	**Androgen**	**Antihypertensor**
Novobiocin sodium salt	Testosterone propionate	Ajmaline
β-lactams	**Antiarrhythmic**	Nisoldipine
Ampicillin trihydrate	Amiodarone hydrochloride	**Antimalarial**
Azlocillin sodium salt	**Anthracycline**	Quinacrine dihydrochloride dehydrate
Bacampicillin hydrochloride	Doxorubicin hydrochloride	**Antiprotozoa**
Benzathine benzylpenicillin	**Antibacterial**	Nifurtimox
Benzylpenicillin sodium	Chloramphenicol	**Anxiolytic**
Cefamandole sodium salt	Fusidic acid sodium salt	Harmol hydrochloride monohydrate
Cefazolin sodium salt	Merbromin	**Diuretic**
Cefotiam hydrochloride	Monensin sodium salt	Ethacrynic acid
Cephalothin sodium salt	Thiamphenicol	**Mydriatic**
Cloxacillin sodium salt	**β-lactams**	Yohimbine hydrochloride
Dicloxacillin sodium salt	Amoxicillin	**Vasorelaxant**
Flucloxacillin sodium	Cefaclor	Harmane hydrochloride
Meropenem	Cefepime hydrochloride	
Metampicillin sodium salt	Cefixime	
Nafcillin sodium salt monohydrate	Cefmetazole sodium salt	
Phenethicillin potassium salt	Cefoperazone dehydrate	
Piperacillin sodium salt	Ceforanide	
Talampicillin hydrochloride	Cefotaxime sodium salt	
Ticarcillin sodium	Cefotetan	
Biguanides	Cefoxitin sodium salt	
Alexidine dihydrochloride	Imipenem	
Chlorhexidine	Moxalactam disodium salt	
Fluoroquinolones	**Fluoroquinolones**	
Ciprofloxacin hydrochloride	Lomefloxacin hydrochloride	
Nadifloxacin	Norfloxacin	
Ofloxacin	**Lincosamides**	
Glycopeptide	Lincomycin hydrochloride	
Vancomycin	**Macrolides**	
	Oleandomycin phosphate	
	Spiramycin	
	Troleandomycin	
	Quaternary Ammonium Compounds	
	Benzethonium chloride	
	Dequalinium dichloride	
	Sanguinarine	
	Anticancer	

hydrochloride
Lincosamides
Clindamycin hydrochloride
Macrolides
Dirithromycin
Erythromycin
Josamycin
Roxithromycin
Oligopeptides
Thiostrepton
Quaternary Ammonium Compounds
Methyl benzethonium chloride
Rifamycins
Rifampicin
Tetracyclines
Chlortetracycline hydrochloride
Demeclocycline hydrochloride
Doxycycline hyclate
Meclocycline sulfosalicylate
Methacycline hydrochloride
Minocycline hydrochloride
Oxytetracycline dihydrate
Tetracycline hydrochloride
Antifungal
Butoconazole nitrate
Clioquinol
Econazole nitrate
Isoconazole
Miconazole
Sertaconazole nitrate
Antihelminthic
Niclosamide
Detergents
Thonzonium bromide
Ellipticine
Tamoxifen citrate
Anticholelithogenic
Chenodiol
Lithocholic acid
Anticoagulant
Dicumarol
Phenindione
Antidepressant
Clorgyline hydrochloride
Antidiabetic
Glimepiride
Antifungal
Hexetidine
Ketoconazole
Sulconazole nitrate
Antigonadotropin
Danazol
Antihyperlipoproteinemic
Fluvastatin sodium salt
Antihyperlipidemic
Simvastatin
Antihypertensor
Prazosin hydrochloride
Anti-inflammatory
Aceclofenac
Acemetacin
Diclofenac sodium
Indomethacin
Meclofenamic acid sodium salt monohydrate
Mefenamic acid
Meloxicam
Parthenolide
Sulindac
Tenoxicam
Tolfenamic acid
Antileprosy
Clofazimine
Antimalarial
Primaquine diphosphate
Antineoplastic
Daunorubicin hydrochloride
Antioestrogen
Lynestrenol
Antiviral
Trifluridine

	Coronarodilatator
	Benzbromarone
	Cystic Acne
	Isotretinoin
	Estrogen
	Diethylstilbestrol
	Hypocholesterolemic drug
	Tiratricol, 3,3',5-triiodothyroacetic acid
	Hypolipidemiant
	Fenofibrate
	Keratolytic
	Retinoic acid
	Local anesthesic
	Dyclonine hydrochloride
	Mucolytic
	Tyloxapol
	Vasodilator
	Ketanserin tartrate hydrate
	Perhexiline maleate
	Suloctidil
	Vitamins
	Calciferol
	Menadione
	Vitamin K2
	Other
	Chicago sky blue 6B
	Chrysene-1,4-quinone
	Clomiphene citrate (Z,E)
	Homosalate
	Iopanoic acid
	Methiothepin maleate

[a] no planktonic growth observed at 10 μM
[b] less than 50% of control biofilm at 10 μM
[c] greater than 200% of control biofilm at 10 μM

Table S3.3. Previously approved drugs with effects on established *L. monocytogenes* biofilms

Growth inhibitors of shed biofilm cells[a]	Biofilm dispersants[b]	Biofilm stimulators[c]
Antibacterial	**Anthracycline**	**Antibacterial**
Florfenicol	Doxorubicin hydrochloride	**β-lactams**
Lasalocid sodium salt	**Antibacterial**	Pivampicillin
Trimethoprim	**β-lactams**	**Anticancer**
Aminocoumarin	Cefaclor	Tamoxifen citrate
Novobiocin sodium salt	Cefepime hydrochloride	**Antineoplastic**
β-lactams	Cefmetazole sodium salt	Daunorubicin hydrochloride
Amoxicillin	Cefoperazone dihydrate	**Antiviral**
Ampicillin trihydrate	Cefotaxime sodium salt	Trifluridine
Azlocillin sodium salt	Cefotiam hydrochloride	**Diuretic**
Bacampicillin hydrochloride	Cefuroxime sodium salt	Ethacrynic acid
Benzathine benzylpenicillin	Moxalactam disodium salt	**Estrogen**
Benzylpenicillin sodium	**Fluoroquinolones**	Hexestrol
Cefalonium	Norfloxacin	**Treatment of age-related macular degeneration**
Cefazolin sodium salt	Ofloxacin	Verteporfin
Cephalothin sodium salt	**Quaternary Ammonium Compounds**	**Provitamin**
Cloxacillin sodium salt	Benzethonium chloride	Menadione
Dicloxacillin sodium salt	**Anticoagulant**	
Flucloxacillin sodium	Dicumarol	
Meropenem	**Cystic Acne**	
Metampicillin sodium salt	Isotretinoin	
Nafcillin sodium salt monohydrate	**Keratolytic**	
Phenethicillin potassium salt	Retinoic acid	
Piperacillin sodium salt	**Mucolytic**	
Talampicillin hydrochloride	Tyloxapol	
Ticarcillin sodium		
Biguanides		
Alexidine dihydrochloride		
Chlorhexidine		
Fluoroquinolones		
Ciprofloxacin hydrochloride		
Nadifloxacin		
Glycopeptides		
Vancomycin hydrochloride		

Lincosamides
Clindamycin hydrochloride
Macrolides
Erythromycin
Josamycin
Roxithromycin
Oligopeptides
Thiostrepton
Quaternary Ammonium Compounds
Methyl benzethonium chloride
Rifamycins
Rifabutin
Rifampicin
Tetracyclines
Chlortetracycline hydrochloride
Demeclocycline hydrochloride
Doxycycline hyclate
Meclocycline sulfosalicylate
Methacycline hydrochloride
Minocycline hydrochloride
Oxytetracycline dihydrate
Tetracycline hydrochloride
Antihelminthic
Niclosamide

[a] no planktonic growth observed at 10 μM
[b] less than 50% of control biofilm at 10 μM
[c] greater than 200% of control biofilm at 10 μM

Table S3.4. MICs of β-lactams for different *L. monocytogenes* strains

Test Compound	Minimal Inhibitory Concentrations (μM)[a]			
	LMFI[b] 1/2a	LMFI[b] 1/2b	*L. monocytogenes* 568	*L. monocytogenes* EGD-e
Amoxicillin	0.8	0.8	0.8	0.4
Ampicillin	0.31	0.63	0.31	0.63
Azlocillin	1.25	2.5	2.5	2.5
Cloxacillin	10	10	5	10
Dicloxacillin	2.5	2.5	2.5	2.5
Benzathine Benzylpenicillin	0.16	0.31	0.31	0.31
Penicillin G	0.31	0.63	0.31	0.31
Piperacillin	5	10	5	5
Cefotaxime	>100	100	>100	>100
Cefoxitin	50	50	50	50
Cefixime	>100	>100	>100	>100
Cefmetazole	50	50	50	50
Cefaclor	>10	>10	>10	>10
Cefalonium	1.6	1.6	1.6	1.6
Cefotiam	3.1	3.1	3.1	3.1
Cefuroxime	100	100	100	100

[a] MIC, minimal inhibitory concentration in tryptic soy broth
[b] LMFI, *L. monocytogenes* food isolate

CHAPTER FOUR

DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms

Co-authorship statement

Chapter Four consists of the following publication:

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The contributions of each author are outlined as follow:

1) All experiments were conducted by U.T.N.
2) Manuscript was conceived and written by U.T.N. and L.L.B.

DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms

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Running title: Proteinase K is a highly effective *Listeria* biofilm dispersant

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Abstract

Current sanitation methods in the food industry are not always sufficient for prevention or dispersal of *Listeria monocytogenes* biofilms. Here, we determined if prevention of adherence or dispersal of existing biofilms could occur if biofilm matrix components were disrupted enzymatically. Addition of DNase during biofilm formation reduced attachment (<50% of control) to polystyrene. Treatment of established 72 h biofilms with 100 μg/mL of DNase for 24 h induced incomplete biofilm dispersal, with <25% biofilm remaining compared to control. In contrast, addition of proteinase K completely inhibited biofilm formation, and 72 h biofilms - including those grown under stimulatory conditions - were completely dispersed with 100 μg/mL proteinase K. Generally-regarded-as-safe proteases bromelain and papain were less effective dispersants than proteinase K. In a time course assay, complete dispersal of *L. monocytogenes* biofilms from both polystyrene and type 304H food-grade stainless steel occurred within 5 min at proteinase K concentrations above 25 μg/mL. These data confirm that both DNA and proteins are required for *L. monocytogenes* biofilm development and maintenance, and that these components of the biofilm matrix can be targeted for effective prevention and removal of biofilms.

Introduction

Listeria monocytogenes biofilms in the food industry can pose a serious threat to consumers. Biofilm formation enables *L. monocytogenes* to survive unfavourable environmental conditions including high salt, low pH and low temperature, and to become tolerant of chemicals used for sterilization (43, 256-258). Current sanitation methods are not sufficient to remove *L. monocytogenes*, as demonstrated by recent recalls of food products contaminated with *L. monocytogenes* (2, 79-81). Thus, it is important to find effective ways to remove biofilms.

For biofilms to develop, surface contact followed by a transition to irreversible attachment is necessary. There is a potential role for flagella in biofilm formation of *L. monocytogenes,* particularly in attachment to surfaces (14), but the evidence is mixed. Although there was a decrease in initial attachment for a mutant lacking flagella, eventually it formed hyperbiofilms (57). Once irreversible attachment to a surface occurs, genes involved in cell surface protein expression and extracellular polymeric substance (EPS) production are activated through a process called quorum sensing (QS), used for cell density-dependent communication (62, 63). In *L. monocytogenes*, the peptide-based QS system accessory gene regulator (Agr) modulates surface-dependent responses. In the absence of the response regulator (AgrA) and putative precursor peptide (AgrD), there was a decrease in initial attachment and

biofilm formation compared to the parental strain with the first 24 h. However, the difference in biofilm formation between mutants and wild type was not significant at 48 and 72 h (67). After irreversible attachment, the cells form small microcolonies that will develop into mature biofilms. The EPS of *L. monocytogenes* biofilms is reportedly composed of proteins and nucleic acids (37, 46). Examination of the highly conserved genomes of *L. monocytogenes* strains sequenced to date (259-261) revealed that they lack genes encoding potential polysaccharide biosynthetic enzymes that could be part of the EPS matrix. Borucki and colleagues (70) demonstrated that *L. monocytogenes* could be stained with ruthenium red, suggesting that there were potentially extracellular polysaccharides in the matrix; however, their data were not conclusive, because ruthenium red also binds peptidoglycan and teichoic acids on the cell surface.

The final step in the biofilm cycle is dispersal, but the exact mechanisms by which *L. monocytogenes* biofilms disperse are not known. Unfavourable environmental conditions and shear forces can cause biofilms to disperse into smaller aggregates. Accumulation of toxins, changes in the levels of nutrients, EPS degradation enzymes secreted by prophages, and/or depletion of oxygen can induce biofilm dispersal (62, 73, 74). This tightly regulated process can involve degradation of the EPS matrix, production of surfactants, lysis of a subpopulation of cells, and induction of flagellar motility (62, 73). The release of cells and small

aggregates allow bacteria to colonize new areas, thus restarting the cycle of biofilm development. Alternatively, individual cells can return to the planktonic state (44, 78).

A genome-wide study of *mariner* transposon insertion mutants to identify genes required for *L. monocytogenes* 10403S biofilm formation showed that mutations in genes involved in cell wall biosynthesis and homeostasis, flagellum synthesis, metabolism, and transcriptional regulation significantly reduced biofilm formation (48). Extracellular DNA (eDNA) was suggested to be a major component of the EPS matrix, because addition of 100 μg/mL DNase I decreased attachment and dispersed established biofilms (46). However, treatment with 100 μg/mL proteinase K, a serine protease, did not affect attachment under their experimental conditions, suggesting that proteinaceous adhesins were less important (46). In contrast, Smoot et al. (83) demonstrated that the addition of 0.01% trypsin to the attachment medium resulted in 99.9% reduction of *L. monocytogenes* on Buna-N rubber and stainless steel surfaces, supporting the potential involvement of proteins in surface attachment. In addition, sub-lethal concentrations of serratiopeptidase affected the ability of *L. monocytogenes* to form biofilms and invade host cells, possibly due to reductions in the levels of surface proteins that function as ligands (47).

Here, we investigated which components of the matrix of *L. monocytogenes* 568 were required for biofilm formation and determined if removal of these components could prevent attachment or induce biofilm dispersal. We confirm that extracellular DNA is part of the biofilm matrix, as the addition of DNase I reduced biofilm formation on polystyrene, but did not completely inhibit biofilm development. In addition, proteins were required for attachment to surfaces, because treatment with proteinase K completely abolished biofilm formation — even under biofilm-stimulatory conditions —and induced biofilm dispersal, even at low concentrations. A time-course assay using proteinase K showed that 100% of established biofilms of multiple strains could be removed from polystyrene or stainless steel surfaces within 5 min. These data highlight the aspects of *L. monocytogenes* EPS that should be targeted in the design of effective treatments for removal or prevention of biofilms on a variety of surfaces.

Materials and Methods

Preparation of DNase I, proteinase K, papain, bromelain, and ampicillin

DNase I, proteinase K, papain, bromelain and ampicillin powder stocks were purchased from Sigma-Aldrich. Stock solutions of DNase I, proteinase K, papain (20 mg/mL in sterile Milli-Q water), bromelain (1mg/mL in sterile Milli-Q water), and ampicillin (10 mM in DMSO) were

stored at -20^{o}C and diluted in water or DMSO, respectively, for the initial test concentrations.

L. monocytogenes strains 568, EGD-e, and LMFI 1/2a biofilm formation assays

L. monocytogenes 568 and EGD-e, both of serovars 1/2a, were the gift of Dr. Lisbeth Truelstrup-Hansen (Dalhousie U). A food isolate belonging to serotype 1/2a (*L. monocytogenes* food isolate, LMFI) was provided by Dr. Burton Blais of the Canadian Food Inspection Agency (CFIA - Ottawa, Ontario). *L. monocytogenes* biofilm assays were performed as previously described (59). Briefly, overnight cultures of *L. monocytogenes* were diluted to OD_{600} ~0.03 in TSB. DNase I and proteinase K were added to each well with the culture (1:100 dilution). The biofilms were grown on polystyrene pegs for 72 h (3 passages x 24 h) at 37^{o}C, 200 rpm, and quantified using crystal violet (CV).

GraphPad Prism 5 (GraphPad Software, Inc) was used to generate planktonic cell density and CV absorbance graphs. The one-way analysis of variance (ANOVA) test and Dunnett's post test software packages in GraphPad Prism were used to calculate statistical values (*P*-values) (59).

L. monocytogenes biofilm dispersal assay on polystyrene pegs and food-grade type 304H stainless steel

L. monocytogenes biofilms were grown in the presence of 1% DMSO as control and under stimulatory conditions with sub-minimal inhibitory concentration of ampicillin (0.04 μM) on polystyrene pegs (59). Following 72 h of incubation, the biofilms were washed with 1X PBS for 10 min to remove loosely adhering cells and then treated with 100 μg/mL (final concentration) DNase I or proteinase K at 37^{o}C, 200 rpm, 24 h. Established biofilm were treated with papain (final concentrations 3.1-100 μg/mL) or bromelain (final concentrations 0.31 – 10 μg/mL) at 37^{o}C, 200 rpm, 24 h. Crystal violet (0.1% wt/vol) was used to stain the remaining biofilms on the pegs. In the proteinase K time course biofilm dispersal assay, the pegs were treated with proteinase K (0.8-100 μg/mL final concentration) for 5, 15, 30, or 60 min, 37^{o}C, 200 rpm. Afterwards the biofilms remaining on the pegs were quantified using 0.1% (wt/vol) crystal violet.

L. monocytogenes biofilms grown on type 304H food-grade stainless-steel coupons were washed with 1X PBS for 10 min and then treated with 50-200 μg/mL proteinase K for 5 min. The stainless-steel coupons were prepared for scanning electron microscopy as previously described (59), in duplicate, and remaining biofilm was evaluated by visual inspection of 3 randomly chosen fields of view per coupon.

Results

DNase I and proteinase K treatment reduce biofilm formation on polystyrene

A previously established biofilm assay (59) was used to evaluate the ability of DNAse and proteinase K to prevent biofilm formation on polystyrene surfaces. We also tested the effects of the peptidoglycan-cleaving enzyme lysozyme, but it had no effect on biofilm formation in our assay conditions up to 200 μg/mL (data not shown). In the presence of DNase I (concentration range 6.3 – 200 μg/mL), biofilm formation was ~30-40% compared to control (Figure 4.1a). This level of inhibition was achieved at the lowest concentration tested, and increasing concentrations did not further reduce biofilm formation. In addition, there was slight increase in planktonic cell density at all concentrations tested (~110 to 120% compared to control).

Proteinase K was a more effective biofilm inhibitor than DNase I. At concentrations between 6.3 – 200 μg/mL, proteinase K completely inhibited biofilm formation with a concomitant increase in planktonic cell density (Figure 4.1b). At the lowest concentration of proteinase K tested (0.2 μg/mL), biofilm formation was ~40% of control, while concentrations >0.8 μg/mL resulted in no detectable biofilm (supplementary data Figure S4.1). These data show that proteinase K is a potent biofilm inhibitor.

DNase and proteinase K disperse established biofilms under normal and stimulatory conditions

Previous studies reported that DNase I dispersed established *L. monocytogenes* biofilms at 100 μg/mL in both microtiter plates and flow cell assays (46). As shown in Figure 4.2a, 100 μg/mL DNase I treatment of 72 h biofilms caused dispersal. The amount of established biofilm remaining was ~25% of control, with little effect on planktonic cell density. We found that ampicillin at sub-minimal inhibitory concentrations (sub-MIC) could stimulate biofilm formation (>150% compared to control) (262) and tested whether DNase I could disperse biofilms grown under those stimulatory conditions. Following 24 h of DNase I treatment, there was similarly ~25% of stimulated biofilm remaining compared to the stimulated control (Figure 4.2a).

When established biofilms were treated with proteinase K, both control and ampicillin-stimulated biofilms were reduced to undetectable levels (Figure 4.2b). However, treatment with proteinase K led to a pronounced increase in planktonic cell density (>200% compared to control) (Figure 4.2b). This result suggests that proteins are a key part of the EPS matrix, and/or that proteinase K degrades proteinaceous adhesins that *L. monocytogenes* uses to attach to surfaces, matrix components, or other bacteria, releasing them from the biofilm without

killing the cells. The increased planktonic cell density could also result from higher nutrient availability (peptides and amino acids).

Proteinase K disperses *L. monocytogenes* biofilms in as little as 5 minutes

Proteinase K at 100 μg/mL dispersed established *L. monocytogenes* 568 biofilms, prompting us to measure how rapidly we could achieve complete dispersal. Established biofilms were treated with increasing concentrations of proteinase K (0.8-100 μg/mL) for 5, 15, 30 or 60 min (Figure 4.3a). For 60 min exposures, concentrations ≥1.6 μg/mL completely removed biofilm from the pegs. With decreasing exposure time, the minimal concentration of proteinase K resulting in complete dispersal increased. At concentrations ≥3.1 μg/mL, the amount of biofilm remaining after 15 min was <5%. At concentrations ≥25 μg/mL, 5 min incubation resulted in complete dispersal of established biofilms, while at lower concentrations (0.8-12.5 μg/mL), 20-90% biofilm remained following 5 min of treatment.

To determine if proteinase K can be used as a general *L. monocytogenes* biofilm dispersant, we tested the effect of proteinase K on different *L. monocytogenes* strains, the common EGD-e strain and a food isolate, LMFI (Figures 4.3b and c, respectively). Similarly to *L. monocytogenes* 568, concentrations ≥1.6 μg/mL removed most or all of *L.*

monocytogenes EGD-e biofilm from the pegs (<5% remaining) after 60 min of treatment. After 5 min exposure to proteinase K at concentrations >25 μg/mL, <10% biofilm remained on the pegs (Figure 4.3b). Proteinase K was also effective at removing LMFI biofilm. At concentrations ≥1.6 μg/mL, there was <15% biofilm remaining after 60 min treatment (Figure 4.3c). Following 5 min exposure to proteinase K at concentrations ≥25 μg/mL, <20% biofilm remained compared to control.

We then compared the effectiveness of proteinase K relative to 'generally regarded as safe' (GRAS) protease food additives – bromelain and papain. GRAS proteases are used in the food industry as meat tenderizers (263). Treatment of established *L. monocytogenes* biofilm with 100 μg/mL of papain for 24 h resulted in ~18% biofilm remaining, while ~85% remained at the lowest concentration tested (3.1 μg/mL) (Figure 4.4a). Treatment with bromelain (0.31 – 10 μg/mL, the maximum concentration that was soluble in our assay) did not disperse established biofilms even with prolonged incubation, suggesting GRAS proteases are less effective than proteinase K at dispersing existing biofilms.

Established biofilms on food-grade stainless steel can be dispersed by proteinase K

In addition to testing the effect of proteinase K on *Listeria* biofilms formed on polystyrene, we tested its effects on biofilms formed on type

304H food-grade stainless steel coupons (59). As shown in Figures 4.5a and c, individual cells and small colonies were attached to the coupons under control conditions. Exposure to 50 μg/mL of proteinase K for 5 min dispersed the small microcolonies, with few cells adhering to the surface (Figures 4.5b and d, arrows). Increased concentrations of proteinase K (100 and 200 μg/mL) caused further dispersal (Figures 4.5e and f, respectively). Treatment with proteinase K at 200 μg/mL caused biofilms to disperse completely within 5 min (Figure 4.5f). Thus, proteinase K is a highly effective biofilm dispersant on both plastic and stainless steel.

Discussion

To reduce outbreaks of disease caused by foodborne pathogens, manufacturers must ensure that their food production areas contain minimal numbers of potentially pathogenic microorganisms. Because these microorganisms are typically found in biofilms, they can resist sanitization. To prevent biofilm formation and remove existing biofilms, many studies have focused on the use of chemicals or other strategies that target various steps of biofilm formation (50, 90, 264).

Among the strategies under investigation is the use of food-safe additives. Many FDA-approved products have been shown to delay and/or inhibit *L. monocytogenes* growth. Nisin, a GRAS peptide, inhibits the growth of *L. monocytogenes* by depolarizing the cytoplasmic membrane

(190, 265). However, nisin-resistant mutants have been found at frequencies of up to 10^{-6}, which suggests that use of nisin in the food industry could result in a growing population of nisin-resistant *L. monocytogenes* (266). Select medicinal and culinary herbs (1 mg/mL) when added at the beginning of a *Listeria* biofilm formation assay could reduce biofilm attachment on polyvinyl chloride by at least 50% (50). In contrast, their effects on 4 h old biofilm were less pronounced, with only 3 out of 15 plant extracts reducing the growth of established biofilm by at least 50%; in fact, some had the opposite effect, enhancing biofilm growth (50). Although herbs can prolong the shelf life of food products and prevent biofilm attachment, they do not completely inhibit bacterial growth at refrigeration temperatures, nor are they completely effective at removing biofilm (50, 267).

Although chemicals are widely used to remove biofilms, non-chemical methods have also been tested. Upon 20 min exposure to ultraviolet (405 nm) light at a dose of 168 J cm^{-2}, biofilm monolayers of *Pseudomonas aeruginosa* and *Escherichia coli* on acrylic and glass were completely inactivated, whereas 2.75 and 2.48 log reductions in viability were observed with S*taphylococcus aureus* and *L. monocytogenes*, respectively (268). Some surfaces in the food plants may be inaccessible to UV light, preventing activity against biofilms. Others have reported that

ultrasound treatment – alone or in combination with chemicals – could reduce the amount of bacterial cells on food-contact surfaces (85, 269).

Many of these methods target specific stages of the biofilm formation cycle - either inhibition of attachment or disruption of existing biofilms. Because the biofilm EPS matrix can contain DNA, proteins and/or polysaccharides, we tested if degradation of such components could prevent biofilm formation and/or disperse established biofilms. Because *L. monocytogenes* lacks obvious polysaccharide biosynthesis-encoding genes (4, 46), we focused on DNA and proteins.

DNase I has been reported to block or alter biofilm formation and morphology in both Gram-positive and Gram-negative bacteria, including *S. pneumoniae*, *S. aureus, E. coli*, and *P. aeruginosa* (270-272). The morphology of biofilms differs among species; *L. monocytogenes* biofilms can be small microcolonies, a homogeneous layer of cells, or ball-shaped microcolonies surrounded by a network of knitted chains (70-72).

When *L. monocytogenes* biofilms were formed in the presence of ≥6.3 μg/mL DNase I, biofilm formation was ~30-40% compared to the untreated sample, suggesting extracellular DNA (eDNA) is important for attachment (Figure 4.1a). Similarly, Harmsen et al. (2010) tested the effect of higher concentrations – 100 μg/mL DNase I – on 41 *L. monocytogenes* strains and demonstrated that eDNA is required for biofilm attachment and development (46). In their work, DNase I was added at different time

points after inoculation (0, 9, 24 and 48 h) and biofilms were quantified after 51 h. Addition of DNase I at early time points (0-24 h after inoculation) reduced adherence and biofilm formation in a strain-dependent manner. After 51 h, <10% biofilm remained, a more pronounced effect than our results at the same concentration. The differences could relate to differences in growth media used, and/or the assay format. Our assay involves passage of biofilm 3 x 24 h into fresh TSB with compound, a method initially developed to provide robust Z' scores for small molecule screening (59), whereas Harmsen et al. had quantifiable biofilm following 51 h of incubation at 37^{o}C without passage. DNase I was shown previously to both inhibit and disperse *S. aureus* biofilms, but it was not effective for *S. epidermidis* biofilms (273). Together, these results suggest that EPS composition can vary between closely related strains and species, and with growth conditions, thus empirical testing is necessary to define the most important matrix components.

In addition to preventing biofilm attachment, Harmsen et al. showed that treatment of *L. monocytogenes* EGD-e and 412 biofilms on glass with DNase I for 18 h removed >80% of the biomass (46), consistent with our data. Both normal and stimulated 72 h *L. monocytogenes* 568 biofilms treated with 100 μg/mL DNase I for 24 h were partially dispersed, with ~25% biofilm remaining (Figure 4.2a).

Because DNase I treatment neither completely inhibited biofilm formation nor dispersed established biofilms, we examined other adhesive components in *Listeria* EPS. In the presence of proteinase K, no biofilm formed on polystyrene at any of the concentrations tested (6.3 – 200 μg/mL; Figure 4.1b). In contrast, Harmsen et al. saw no significant effect of proteinase K on *L. monocytogenes* EGD-e attachment using cover glass cell culture chambers (46). The differences may depend on the type of surface and/or *Listeria* strains used in the study. In studies of *S. aureus* biofilm formation, initial attachment of strain V329, which expresses biofilm associated protein (Bap) on its surface, was reduced upon proteinase K treatment, whereas the attachment of strain M556, which lacks Bap, was unaffected (274).

Protease treatment has been reported to impair biofilm development or to induce dispersal in *L. monocytogenes, S. aureus, P. fluorescens* and *B. cereus* (47, 83, 254, 274). Treatment of biofilms grown under non-stimulatory and stimulatory conditions on polystyrene pegs with 100 μg/mL proteinase K for 24 h caused complete dispersal without killing the planktonic cells (Figure 4.2b), suggesting that proteins within the biofilm matrix or on the cell surface are required for adhesion. The nature of the *L. monocytogenes* proteinaceous adhesins targeted by proteinase K treatment are under investigation; candidates include the Ami4b autolysin, internalin B, and ActA (Longhi et al., 2008).

Proteinase K was previously reported to be an effective dispersant for *L. monocytogenes* clinical isolates (37). In that work, 1 mg/mL proteinase K – notably, over 100 times more than the lowest concentration tested here – dispersed 84-98% of established biofilm within 3 h at 37^{o}C, depending on the strain. Here we showed that compared to FDA-approved GRAS proteases papain and bromelain, proteinase K was the most effective and could be considered for use as a biofilm dispersant. There was ~18% *L. monocytogenes* 568 biofilm remaining after treatment with 100 μg/mL papain for 24 h while bromelain was ineffective at the concentrations tested (Figure 4.4).

Food contact surfaces in approved manufacturing facilities are routinely inspected to determine if potential pathogens are present. If bacteria are present as highly adherent biofilms, it may be more difficult to obtain representative samples by swabbing. Therefore, it would be useful to have an effective 'spray-on' reagent that could quickly dissociate existing biofilms, potentially allowing for more representative numbers of live cells to be recovered, providing more accurate sampling data. *L. monocytogenes* form biofilms on various materials in food plants, including polypropylene, glass and type 304H food-grade stainless steel (275, 276). Proteinase K concentrations ≥1.6 μg/mL completely dispersed *L. monocytogenes* 568 and EGD-e biofilm within 60 min (Figures 4.3a and b, respectively). At the shortest treatment time tested (5 min), concentrations

≥25 μg/mL proteinase K completely dissociated the biofilm. Proteinase K treatment for 5 min was slightly less effective with the LMFI strain (Figure 4.3c), although <10% biofilm remained after 5 min treatment at concentrations ≥50 μg/mL. Proteinase K was also effective at dispersing *L. monocytogenes* biofilm at room temperature within 5 min to levels comparable to 37°C treatment (data not shown). As shown in Figure 4.5f, 5 min treatment with 200 μg/mL proteinase K also completely removed biofilms from food-grade stainless steel. Based on these data, we suggest that proteinase K could be used for biofilm dispersal, and the effective concentration and contact time required to achieve >80% dispersal can be far shorter than the 3 h reported by Franciosa et al. (Franciosa et al., 2009).

To extend the applicability of our findings, further studies are required to determine the effect of the EPS-degrading enzymes on mixed-species biofilms, which are likely to be present in food processing environments. DNase I has been shown to disperse mixed-species biofilms composed of *S. epidermidis* and *Candida albicans* (277). At 1.25 mg/mL DNase I (notably, over 5x the maximum concentration tested here), both mono-species *S. epidermidis* and mixed-species mature biofilms were dispersed to similar levels. DNase I was less effective at dispersing mixed-species compared to mono-species biofilms at concentrations <1.25 mg/mL (277). It is possible that use of proteinase K,

alone or subsequent to DNase I treatment, could improve dispersal. In conclusion, the results of this work suggest that proteinase K is an effective biofilm inhibitor/dispersant that can be used alone or in conjunction with current methods used to address *L. monocytogenes* contamination.

Acknowledgements

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Figure 4.1

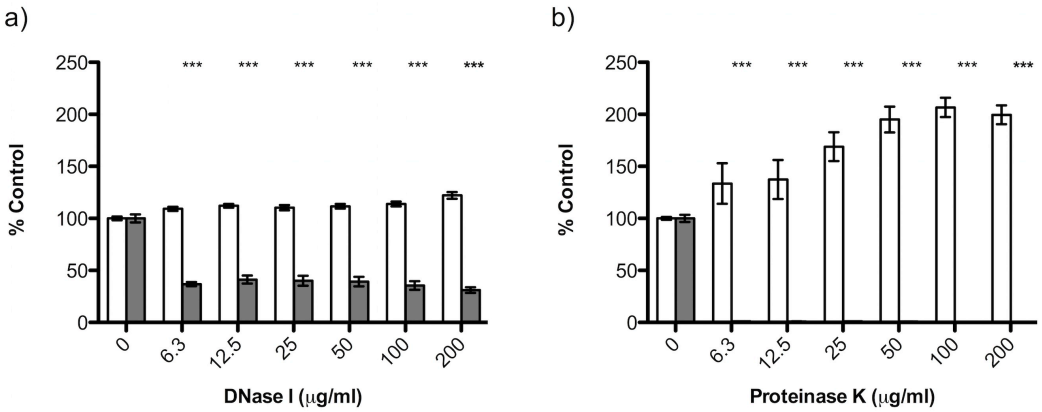
a)
b)
% Control
250
200
150
100
50
0
***
0
6.3
12.5
25
50
100
200
DNase I (μg/ml)
Proteinase K (μg/ml)

Figure 4.1. Inhibition of *L. monocytogenes* 568 biofilm formation on polystyrene by DNase I and proteinase K. *L. monocytogenes* biofilms were grown on polystyrene pegs in the presence of (a) DNase I and quantified using crystal violet staining. Increasing concentrations of DNase I (>6.3 μg/mL) does not reduce more biofilm formation compared to low concentrations. Similarly, in the presence of (b) proteinase K, low concentrations (6.3 μg/mL) can completely inhibit biofilm formation. Planktonic growth at day 3 (white bars) and biofilm formation (grey bars), expressed as a percentage of control (n=4, with standard error shown). ***, $P< 0.001$.

Figure 4.2

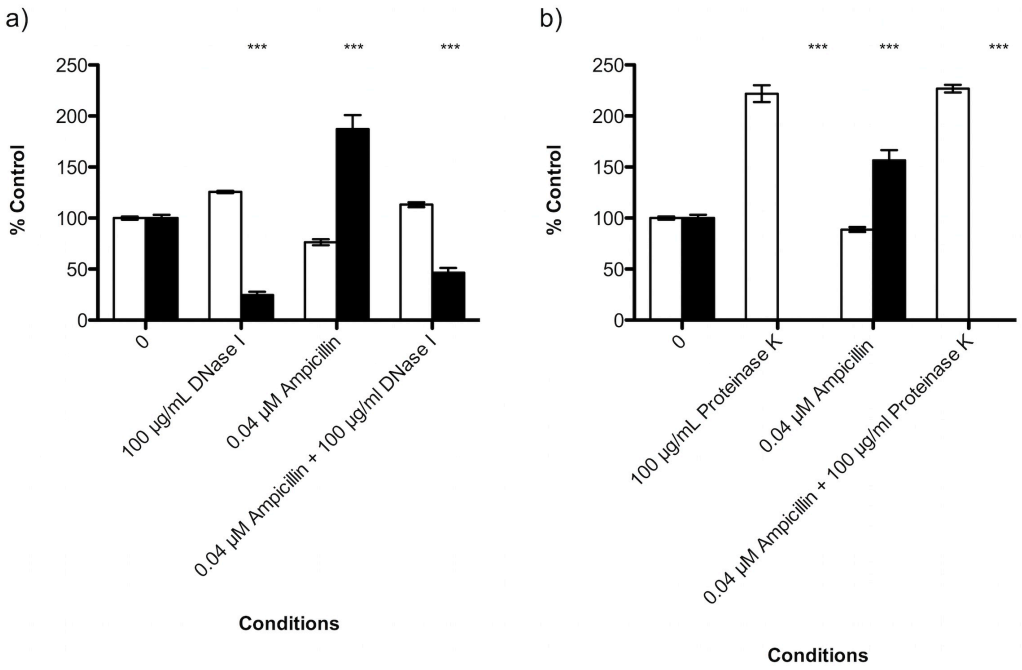
a)
***
***
***
250
200
150
100
50
0
% Control
0
100 µg/mL DNase I
0.04 µM Ampicillin
0.04 µM Ampicillin + 100 µg/ml DNase I
Conditions
b)
***
***
***
250
200
150
100
50
0
% Control
0
100 µg/mL Proteinase K
0.04 µM Ampicillin
0.04 µM Ampicillin + 100 µg/ml Proteinase K
Conditions

Figure 4.2. DNase I and proteinase K disperse established biofilms on polystyrene. *L. monocytogenes* biofilms were grown on polystyrene pegs for 3 d in the absence or presence of ampicillin then subjected to (a) DNase I or (b) proteinase K for 24 h. The amount of biofilm that remained on the pegs after treatment was quantified by crystal violet staining. Planktonic growth at day 4 (white bars) and biofilm that remained on the pegs (black bars) are expressed as a percentage of control (n=3). ***, $P < 0.001$.

Figure 4.3

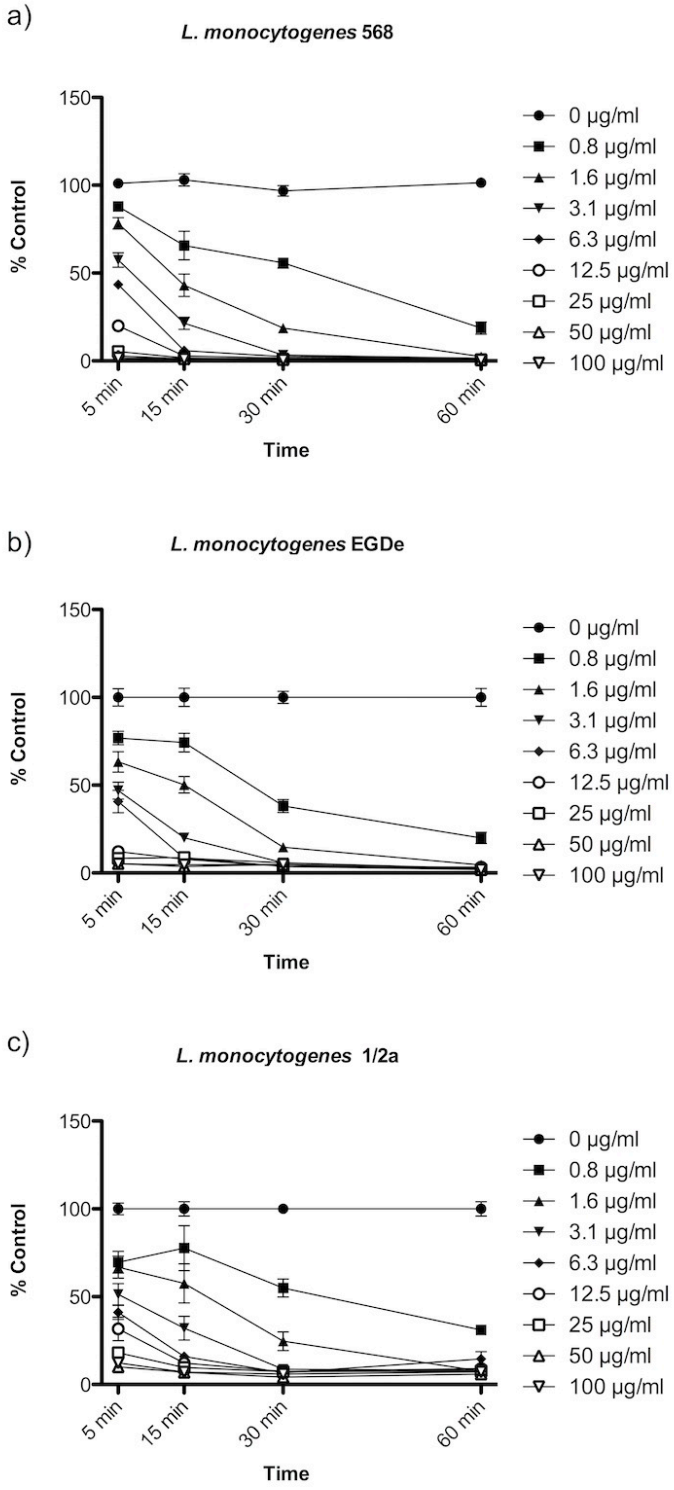
a)
L. monocytogenes 568
% Control
150
100
50
0
5 min
15 min
30 min
60 min
Time
0 µg/ml
0.8 µg/ml
1.6 µg/ml
3.1 µg/ml
6.3 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
b)
L. monocytogenes EGDe
% Control
150
100
50
0
5 min
15 min
30 min
60 min
Time
0 µg/ml
0.8 µg/ml
1.6 µg/ml
3.1 µg/ml
6.3 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
c)
L. monocytogenes 1/2a
% Control
150
100
50
0
5 min
15 min
30 min
60 min
Time
0 µg/ml
0.8 µg/ml
1.6 µg/ml
3.1 µg/ml
6.3 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml

Figure 4.3. Time-course assay of *L. monocytogenes* 568, EGD-e, and LMFI biofilm dispersal by proteinase K. *L. monocytogenes* (a) 568, (b) EGD-e, and (c) LMFI biofilms were grown on polystyrene pegs for 3 d then treated with proteinase K for 5, 15, 30 or 60 min. The amount of biofilm that remained on the pegs after treatment was quantified by crystal violet staining (n=3).

Figure 4.4

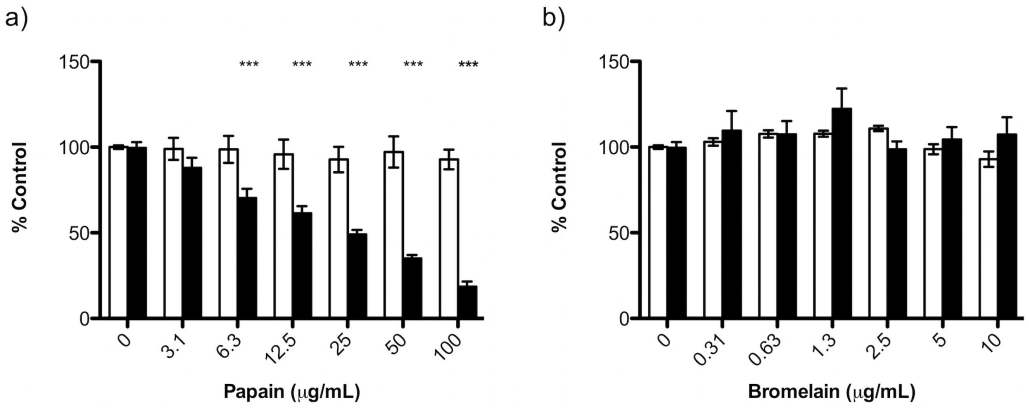
a)
b)
150
100
50
0
% Control
***
0
3.1
6.3
12.5
25
50
100
Papain (μg/mL)
0.31
0.63
1.3
2.5
5
10
Bromelain (μg/mL)

Figure 4.4. The effects of GRAS proteases (papain and bromelain) on established *L. monocytogenes* 568 biofilm. *L. monocytogenes* biofilms were grown on polystyrene pegs for 3 d in 1% DMSO then subjected to (a) papain or (b) bromelain for 24 h. The amount of biofilm that remained on the pegs after treatment was quantified by crystal violet staining. Planktonic growth at day 4 (white bars) and biofilm that remained on the pegs (black bars) are expressed as a percentage of control (n=3). *** $P < 0.001$.

Figure 4.5

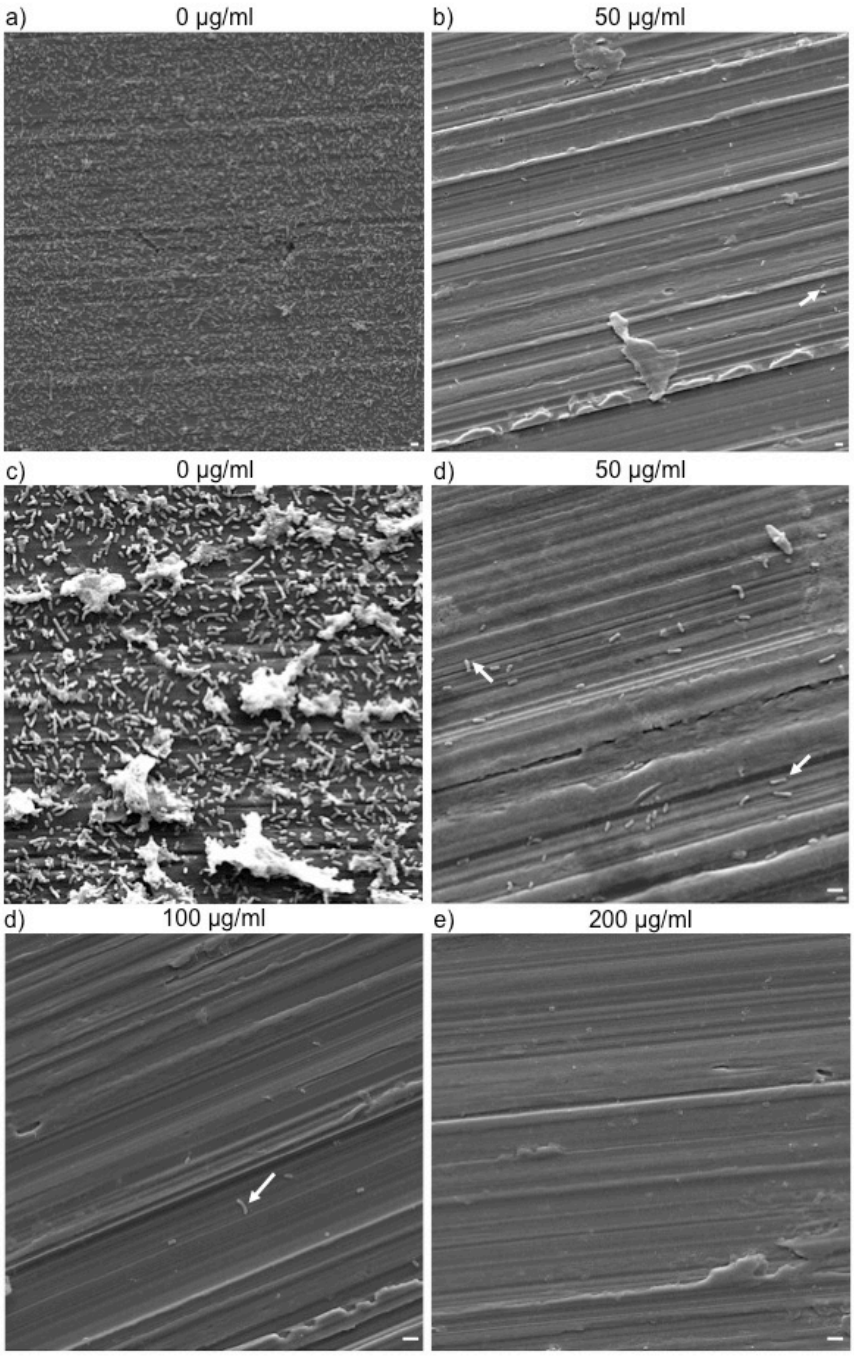

Figure 4.5. Dispersal of established biofilms on food-grade type 304H stainless steel by proteinase K. (a-f) Representative SEM images of *L. monocytogenes* biofilms on food-grade stainless-steel following treatment with proteinase K at various concentrations. Arrows show individual cells that remain attached after treatment. Bar: 2 μm. Magnification: (a-b) 2500x and (c-f) 5000x.

Figure S4.1

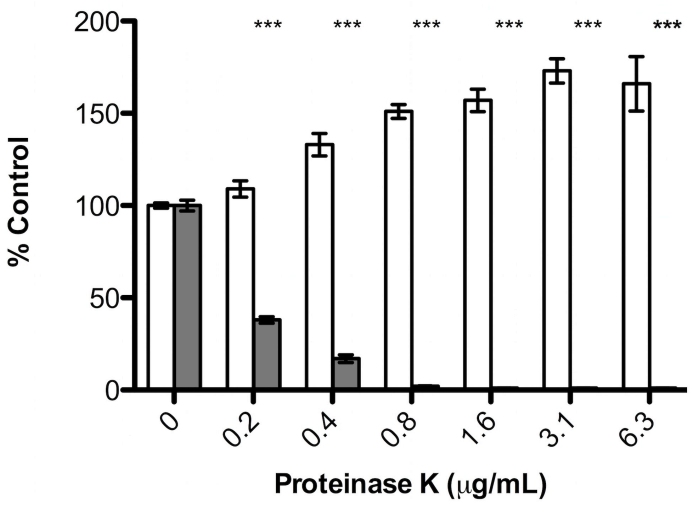

Figure S4.1. Inhibition of *L. monocytogenes* 568 biofilm formation on polystyrene by proteinase K. *L. monocytogenes* biofilms were grown on polystyrene pegs in the presence of proteinase K and quantified using crystal violet staining as described in the Methods. At 0.2 μg/mL proteinase K, biofilm formation was ~40% of control, while higher concentrations resulted in complete inhibition. Planktonic growth at day 3 (white bars) and biofilm formation (grey bars), expressed as a percentage of control (n=2 with standard error shown). ***, $P < 0.001$.

CHAPTER FIVE

Summary and Conclusions

Overview of findings

The data presented here provide insights into small molecules and enzymes that could be used to prevent *L. monocytogenes* biofilm development and/or to disperse established biofilms. These molecules – with different mechanisms of action – affected the same stages of biofilm formation, demonstrating that *L. monocytogenes* biofilm formation is a complex process.

To study *L. monocytogenes* biofilm development, a *L. monocytogenes* biofilm assay that was suitable for high-throughput screening was developed. As a proof of principle, this assay was first used to screen a library of 80 eukaryotic kinase inhibitors to identify compounds that can prevent biofilm formation. Many kinase inhibitors reduced biofilm formation at sub-MIC levels (Figure S2.1). Specifically, palmitic acid-derived PKC inhibitors, palmitoyl-D,L-carnitine and sphingosine, inhibited and/or reduced biofilm formation at sub-MIC levels on polystyrene and food-grade type 304H stainless steel (Figures 2.1 and 2.2). These PKC inhibitors reduced biofilm formation of *L. monocytogenes,* plus *S. aureus* (Figure 2.4), *P. aeruginosa*, and *E. coli*, albeit through different mechanisms (209). The broad-spectrum biofilm inhibitory properties of the PKC inhibitors demonstrate the potential use of these compounds as antibiofilm agents.

Not only is it important to prevent biofilm formation – especially on medical devices and food contact surfaces to prevent recurrence of an infection or future food-borne outbreak – it is also important to disperse established biofilm and prevent the released cells from growing and forming another biofilm. A library of off-patent small molecules whose bioavailability and safety in humans are known was screened to identify compounds that could disperse biofilms and prevent their formation. From this screen, many β-lactams that inhibited biofilm development and/or dispersed established biofilms were identified. However, dose-response assays showed that select β-lactams stimulated biofilm formation at sub-MIC levels (Figures 3.1 and S3.1). The contrasting phenotype observed at high concentrations (killing) versus low concentrations (biofilm stimulation) is called hormesis, a property of all bioactive molecules (97, 98). In our experiments, the stimulatory effect observed was dependent on PBPD1, as the stimulatory β-lactams were unable to induce biofilm formation of a *pbpd1* mutant to the same levels as wild type (Figure 3.5). In addition, the β-lactams displayed biofilm dispersal properties (Figures 3.2 and S3.2), and reduced the growth of cells shed from the biofilm. β-lactams would not be used in the food industry as a biofilm dispersant because of people with allergies to penicillins (32, 40) and the use of antibiotics in that context could lead to resistant bacteria that can enter the food chain (278, 279).

However, understanding how they induce dispersal would aid in identifying new strategies for removing biofilms.

To determine what EPS components were increased under β-lactam stimulatory conditions, EPS-grading enzymes were used on stimulated biofilm to test if they could disperse the biofilm. The EPS of *Listeria* is less well characterized than that of other bacteria. Harmsen and coworkers (46) reported that eDNA was a key component of *L. monocytogenes* EPS, while proteins were less critical, as enzymatic removal of proteins did not affect attachment. However, other studies have demonstrated that proteins are required for surface attachment and/or biofilm formation in *L. monocytogenes* (47, 83). *L. monocytogenes* biofilms grown in the presence of either DNase I or proteinase K were reduced compared to control (Figure 4.1). In addition, both dispersed established biofilms, but did not kill the cells released (Figures 4.2 and 4.5). These data suggest that eDNA is not the only component of *L. monocytogenes* EPS, as 5 min exposure to proteinase K dispersed >90% of biofilms of different *L. monocytogenes* strains (Figure 4.3).

Future Directions

Determining the mode of action for sphingosine

Sphingosine inhibited biofilm formation of *L. monocytogenes, S. aureus*, *P. aeruginosa*, and *E. coli* (Figures 2.1 and 2.4) (59, 209).

Sphingosine inhibits the growth of many food-borne pathogens (201, 219) and is thought to prevent intestinal colonization by these bacteria by binding to the intestinal mucosa (217). In humans, sphingosine has been shown to have many potential health benefits. *S. aureus* colonization on the skin is inhibited by sphingosine (216), and sphingosine derivatives can inhibit early stages of colon cancer (280) and cholesterol absorption (281). However, its mechanism of action on bacteria has not been characterized.

Using a concentration gradient plate-based assay, we selected for mutants that are resistant to sphingosine (2-8X MIC) in *L. monocytogenes* 568 and EGD-e. The genome of *L. monocytogenes* 568 has been sequenced, but not fully assembled. However, *L. monocytogenes* EGD-e has been sequenced and extensively studied compared to *L. monocytogenes* 568. The *L. monocytogenes* 568 and EDG-e mutants resistant to sphingosine could be sequenced via the Illumina MiSeq Sequencing System to identify single nucleotide polymorphisms (SNPs) within the genome. Based on previously published analyses of *Listeria* genomes (3, 282), diversity is low and thus few SNPs are expected. PCR-based screening showed that of 13 isolates of 1/2a serotype, compared to a hypothetical common ancestor, one had 27 SNPs, whereas the others had 0-1 SNPs (3). Another study showed 11 SNPs in the genome sequence of 4 isolates, and a maximum of 8 SNPs between any two strains. These isolates contaminated a food production facility between

1988-2000 (282). Once the SNPs related to acquisition of sphingosine resistance are identified, future studies will be directed at verifying resistance through mutating the gene(s) in the parental strain.

An alternative approach to whole genome sequencing is to screen a *L. monocytogenes* 568 transposon mutant library (283) against sphingosine at 25 μM (2X MIC) to identify potential sphingosine-resistant transposon mutants. Discovery of these mutants could reveal certain genes involved in resistance pathways; however, this approach is limited by the possibility that resistance may require multiple mutations in the genome. Such experiments have the potential to identify the genes involved in resistance, which may indicate the pathway sphingosine uses to inhibit biofilm formation/ planktonic growth. However, it may be that many genes are involved.

The effectiveness of proteinase K on mixed-species biofilms

As shown in Figure 4.3, proteinase K can disperse *Listeria* mono-species biofilms efficiently. However, in most environments, mixed-species biofilm predominate (52, 284). In the food industry, *L. monocytogenes* would most likely colonize surfaces and form biofilms with other bacteria. To determine if proteinase K is effective on mixed-species biofilms, *L. monocytogenes* will be grown with other bacteria that have been shown to form biofilms with *Listeria,* such as *Salmonella enterica* and *Lactobacillus*

plantarum (285, 286) on different surface types. Following biofilm establishment, the biofilms will be treated with varying concentrations of proteinase K and treatment time to determine if proteinase K is effective at removing the mixed-species biofilm. To quantify the amount of biofilm remaining, CV staining will be completed. Alternatively, the amount of biofilm attached following treatment can be visually inspected via SEM.

Analysis of the cell surface proteome of β-lactam treated cells

Many factors aid in bacterial attachment to abiotic and biotic surfaces, including surface proteins (43, 52, 78). The proteins required for attachment are not well characterized in *L. monocytogenes*. We have demonstrated that β-lactams prevented biofilm formation and induced biofilm dispersal (Figures 3.1, 4.2, S3.1 and S3.2). We hypothesize that inhibition of HMW PBPs activity by β-lactams affect the display of sortase-dependent adhesins that are incorporated into the cell wall during PG synthesis.

Among the approaches that can be used to determine which proteins are present or absent following treatment is bacterial cell 'shaving', a technique that removes exposed portions of surface proteins of intact cells using trypsin. Following treatment, the supernatant containing peptides is collected via centrifugation and then dialysed to collect the peptides from the supernatant. The peptides are then analyzed

via LC-MS/MS and to prevent false positives, the supernatant of trypsin-free control cells is digested and analyzed via LC-MS/MS (287, 288). *L. monocytogenes* cells will be treated with β-lactams and then subjected to trypsin shaving and LC-MS/MS analysis. The profiles of treated and control cells would be compared to identify differences in the surface proteins. Follow up studies will involve deletion of the surface proteins that were found to be absent in the treated cells to determine which are required for attachment.

An alternative approach to using whole cells is to use insoluble cell wall extract that contains PG and proteins that are strongly associated to the cell wall. Similarly to bacterial cell shaving, the cell wall extract is digested with trypsin and analyzed using 2D nanoliquid LC-MS/MS (147). This protocol has been used to analyze the cell wall proteome of different *Listeria* species and allowed identification of LPXTG-motif proteins that were not identified using other types of methods (147).

Determine if β-lactams induce the release of teichoic acids

In addition to surface proteins, TAs are abundant on the *Listeria* cell surface. β-lactams induce the release of TAs from many bacteria including *S. aureus*, *S. pneumoniae*, and *S. sanguis* (289-291), which could result in a decease in adherence. In *S. aureus,* mutation of *tagO*, which encodes for an enzyme that transfers NAG phosphate to bactoprenyl phosphate

(first step of WTA biosynthesis), resulted in decreased primary attachment and biofilm formation (292). Mutations of genes involved in *L. monocytogenes* TA synthesis and modification such as D-alanylation and synthesis of the glycolipid linker have been shown to decrease biofilm formation by 72-85%, suggesting a role for TAs in *Listeria* biofilm development (48). An enzyme-linked immunosorbent assay (ELISA) will be used to determine if β-lactams induce the release of TA (289, 293). This protocol has been used to detect the release of teichoic acid by antibiotics in *S. aureus* (293). In order to do the ELISA, an antibody would be raised against either the glycerol or ribitol-phosphate moiety of the TA, and purified TA will be required to generate a standard curve. The cells will be grown in the presence or absence of β-lactams and then centrifuged to collect the supernatant, which will be used in the ELISA to determine the amount of TA released (293).

Transcriptome analysis of β-lactam-stimulated biofilm.

Sub-inhibitory concentrations of antibiotics have been shown to induce biofilm formation in many bacteria. In *S. epidermidis*, sub-inhibitory concentrations of tetracycline induce biofilm formation through increasing *ica* expression (105). The *ica* operon encodes genes required for biofilm polysaccharide biosynthesis. In another study, aminoglycosides at sub-MIC levels simulated biofilm formation in both *P. aeruginosa* and *E. coli*

(215). We have demonstrated that sub-MIC β-lactams induce biofilm formation in *L. monocytogenes* and that this stimulatory effect partially depends on PBPD1 (Figure 3.5). Since the *pbpD1* mutant can still form more biofilm compared to control under stimulatory conditions, other factors are involved in biofilm formation.

To determine the genes involved in biofilm formation/ stimulation, RNA-sequencing (RNA-seq) can be used to detect differences in gene expression of *L. monocytogenes* grown in the absence and presence of sub-MIC β-lactams. RNA-seq is a tool to analyse the entire transcriptome and gene expression levels (294, 295). The transcriptome of *P. aeruginosa* and *Aspergillus fumigatus* during planktonic and biofilm growth have been studied using RNA-seq to understand how the expression levels of genes differ between the two growth phases (110, 296). In *L. monocytogenes*, RNA-seq was used to compare the transcriptome of the parental strain to a *sigB* mutant, which is a regulator of genes involved in stress response in order to define the stationary phase stress response transcriptome (294). The transcriptome of biofilms grown in the presence of β-lactams will be compared to control to determine which genes are upregulated and downregulated under biofilm inducing conditions. The results of RNA-seq may provide insights into the genes and pathways are involved in biofilm formation, which can potentially lead to design of drugs that can inhibit biofilm formation that are not toxic to humans.

Significance and Conclusions

Biofilm formation is advantageous to bacteria, protecting them from stressful environmental conditions and allowing the spread and transmission of bacteria, thereby facilitating their survival (2, 44, 297). We have demonstrated using an optimized *Listeria* biofilm assay that small molecules (kinase inhibitors and β-lactams) killed *L. monocytogenes* and/or inhibited biofilm formation on multiple types of surfaces. Although these molecules were initially identified as planktonic growth/ biofilm formation inhibitors, many of them with similar structures had the opposite effect and stimulated biofilm development. In addition, we demonstrated that besides eDNA, proteins are essential to biofilm formation. Proteinase K blocked biofilm formation and disperse established biofilms of *L. monocytogenes* food isolates and lab strains and was more effective than current GRAS proteases. Understanding how *L. monocytogenes* biofilm formation occurs and how small-molecules can alter biofilm development can potentially lead to new methodologies of preventing and dispersing *L. monocytogenes* biofilms.

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