Response of Listeria monocytogenes and Staphylococcus aureus to host defense peptides and behavior in eukaryotic cells

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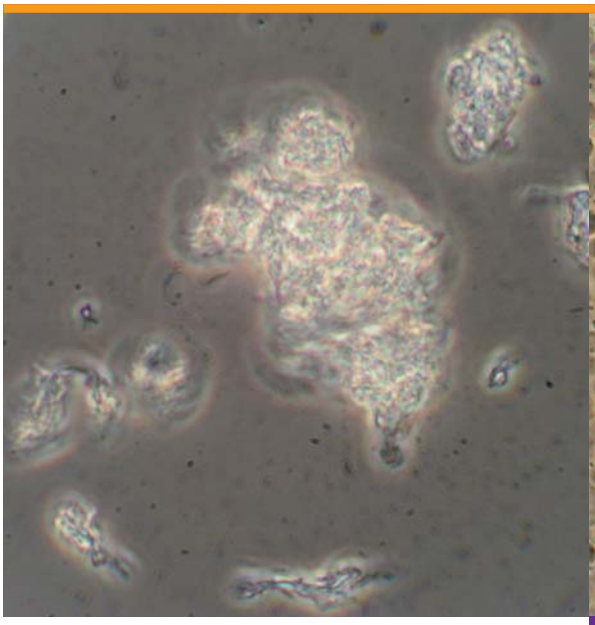

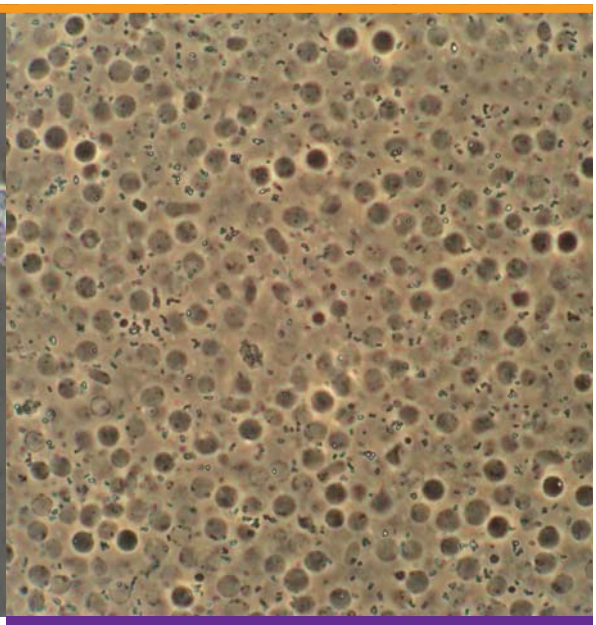

Caroline Trebbien Gottlieb
PhD Thesis
2009

DTU Food
National Food Institute

Response of *Listeria monocytogenes* & *Staphylococcus aureus* to host defense peptides and behavior in eukaryotic cells

Ph.D. thesis

by

Caroline Trebbien Gottlieb

2009

National Food Institute

Technical University of Denmark

Division of Seafood Research

Front page photos:

Left: *Listeria monocytogenes* after treatment with protamine

Right: *Staphylococcus aureus* in human whole blood

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Preface

The work presented in this thesis describes the results obtained during a Ph.D. study following the Ph.D. programme at the Technical University of Denmark in the period from September 2005 to November 2009.
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The work has been carried out at:

- The Technical University of Denmark, National Institute of Aquatic Resources, Department for Seafood Research, DK-2800 Kgs. Lyngby, Denmark
- University of Copenhagen, Faculty of Life Sciences, Department of Veterinary Disease Biology, DK-1870 Frederiksberg, Denmark.

I wish to thank my supervisors, Professor Lone Gram and Professor Hanne Ingmer for their support and ideas given throughout the study. I would especially like to thank Lone for her constructive advices and her tremendous help during all phases of this study.

Also, I would like to thank Line Elnif Thomsen for her help and advices on the work with the transposon mutants and Marianne Halberg Larsen for her help and advices on the work with the cell models.

I would also like to thank the microbiology group at DTU Aqua for providing a nice atmosphere and a stimulating scientific environment. Especially, I would like to thank Anne Holch for our many discussions on every thinkable subject concerning *Listeria* and our collaboration on the virulence studies. I would also like to thank Matthias Wietz for proof-reading my thesis. Also a thank you to the people in the lab at KU-Life for making me feel at home during my stay there.

Finally I wish to thank my friends and my family for their continuous support and encouragement. Especially I would like to thank Kristian and my daughter Thea who have helped me to focus when I needed it and have provided me with all the distraction I could ever wish for whenever that was appropriate.

Caroline Trebbien Gottlieb

Kgs. Lyngby, November 2009

The thesis is based on the following papers:

Paper 1

Caroline Trebbien Gottlieb, Line Elnif Thomsen, Hanne Ingmer, Per Holse Mygind, Hans-Henrik Kristensen, Lone Gram (2008). Antimicrobial peptides effectively kill a broad spectrum of *Listeria monocytogenes* and *Staphylococcus aureus* strains independently of origin, sub-type, or virulence factor expression. *BMC Microbiology*. 8:205.

Paper 2

Caroline Trebbien Gottlieb, Lone Gram (2009). The sensitivity of *Listeria monocytogenes* to host defense peptides is not influenced by innate or environmental stresses or by a key stress regulatory gene. *Journal of Applied Microbiology*. Ready for submission, pending approval by Sanofi-Aventis.

Paper 3

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Paper 4

Anne Holch¤, Caroline Trebbien Gottlieb¤, Marianne Halberg Larsen, Hanne Ingmer, Lone Gram (2009). A group of food processing persistent *Listeria monocytogenes* strains are poor invaders in trophoblastic cells but form normal plaques in fibroblastic cells despite *actA* deletion. *Applied and Environmental Microbiology*. Submitted.
¤ A.H. and C.T.G. contributed equally to this study.

Summary

Response of *Listeria monocytogenes* and *Staphylococcus aureus* to host defense peptides and behavior in eukaryotic cells

The innate immune system constitutes the first line of defense against invading pathogenic microorganisms. Antimicrobial peptides (AMPs) or host defense peptides (HDPs) have constituted a major group of antimicrobial effector molecules of the innate immune defense of all living organisms for millions of years. Hence the development of resistance is considered unlikely. In addition they have been shown to possess immunomodulatory functions that serve to stimulate both innate and adaptive immune responses. This has prompted a massive interest in HDPs as novel antimicrobials. On the other hand, a cardinal feature of pathogenic microorganisms is the ability to resist the actions of these HDPs in order to establish an infection. Hence, the effectiveness of HDPs as well as the virulence of the pathogenic microorganisms are a result of the complex interplay between the microorganisms and the host. In order to evaluate the potential role of HDPs as novel antimicrobials, an understanding of the natural variation in tolerance within bacterial populations and the relation to virulence potential as well as the influence of environmental factors on response to HDPs is needed. In addition an assessment of the potential risk of development of resistance when HDPs are used outside of their natural environments is necessary.

The purpose of this Ph.D. study was to investigate the response of two related Gram-positive pathogenic bacteria, *Listeria monocytogenes* and *Staphylococcus aureus*, to HDPs. We hypothesized that increased virulence could at least in part be explained by increased tolerance to the antimicrobial components of the innate immune system. We examined a collection of 25 *L. monocytogenes* and 16 *S. aureus* strains representing different subtypes, origins and phenotypic behavior. We used four model peptides representing each of the three structural classes of HDPs. Protamine is a linear peptide rich in proline and arginine, plectasin is a fungal defensin, and novispirin G10 and its derivate novicidin are linear α-helical peptides that also belong to the group of cathelicidins. We found that the *L. monocytogenes* and *S. aureus* strains were within each species equally sensitive to HDPs and that this was not paralleled by their phenotypic behavior. Hence, the potential therapeutic use of HDPs is not hampered by naturally occurring resistant bacteria. Also, the environmental conditions that a pathogen is exposed to prior to infection can influence the physiological state of the pathogen and affect the ability to cause infection. We hypothesized

that exposure to food-related stresses (5% NaCl, pH 5.5 and 5°C) would elicit a stress response in *L. monocytogenes* leading to increased tolerance to HDPs. We did not observe an altered tolerance to HDP treatment after exposure to each of the three stress conditions.

Finally, to investigate if resistance to HDPs could be provoked by single mutations we created transposon mutants in both *L. monocytogenes* and *S. aureus* and screened for mutants with increased tolerance to plectasin. We identified a mutant in *S. aureus* with the transposon inserted into the response regulator *hssR* of the two-component system hssRS. This mutant had a two to four-fold increased tolerance to plectasin. We did not identify any resistant mutants in *L. monocytogenes*, suggesting that development of resistance through single mutations is unlikely.

Together, we have shown that natural tolerance to HDPs does not occur in two populations of *L. monocytogenes* and *S. aureus* and that exposure to food-related stresses does not increase the tolerance of *L. monocytogenes* to subsequent HDP treatment. In addition, the development of resistance through single mutations does not seem to occur readily. These results support a potential role of HDPs as novel antimicrobials.

The ability of *L. monocytogenes* to adapt to specific environmental conditions can also lead to the establishment of persistent subpopulations within distinct ecological niches such as food processing facilities. Such strains are likely contaminants of foods and hence from a risk assessment perspective it is important to determine the virulence potential. The *L. monocytogenes* collection used in this study comprises strains of a specific molecular subtype, the RAPD type 9 (random amplified polymorphic DNA) that have been isolated repeatedly from fish processing facilities for a period of several years. It has previously been shown that these strains were low virulent in simple *in vitro* virulence models and non-mammalian models, however in a more complex biological model using pregnant guinea pigs, a RAPD type 9 strain surprisingly infected the placenta and fetuses just as efficiently as a high virulent clinical strain. We hypothesized that the RAPD type 9 strains had an enhanced ability to execute one (or several) of the steps involved in transmission across the placenta and that this might be reflected in differences in sequences of virulence genes important for these steps. We found that the RAPD type 9 strains invaded placental trophoblasts to a lower level compared to clinical strains, and this could be explained by the presence of premature stop codons in *inlA*. Likewise, the ability of the RAPD type 9 strains to spread from cell-to-cell in fibroblasts was not different to clinical strains. We conclude that the RAPD type 9 strains can still be regarded as low virulent with respect to human listeriosis and do not pose a risk to pregnant women and their unborn fetus in particular.

Resumé (in Danish)

Listeria monocytogenes og *Staphylococcus aureus*' respons på antimikrobielle peptider og adfærd i eukaryote celler

Det innate immunsystem udgør det første forsvarsværk mod indvaderende patogene mikroorganismer. Antimikrobielle peptider (AMPer) eller " vært-forsvars-peptider" (host defense peptides, HDPer) har været en central gruppe af antimikrobielle effektormolekyler i alle levende organismers immunforsvar i flere millioner år. Derfor anses det for usandsynligt at der udvikles resistens mod disse peptider. Desuden har de vist sig at have immunomodulatoriske funktioner, der stimulerer både det innate og adaptive immunforsvar. Dette har resulteret i en massiv interesse i HDPer som en helt ny gruppe af antimikrobielle stoffer. Omvendt er en af de vigtigste egenskaber ved patogene mikroorganismer deres evne til at modstå virkningen af HDPer, hvilket gør dem i stand til at etablere en infektion. Som følge heraf er både HDPernes effektivitet og de patogene mikroorganismers virulens et resultat af et komplekst samspil mellem mikroorganismer og værten. For at kunne evaluere HDPers mulige rolle som en ny gruppe antimikrobielle stoffer, er det nødvendigt at kende til den naturlige variation i tolerance i en population af patogene bakterier og om en sådan variation kan reflekteres i deres virulenspotentiale. Desuden er det vigtigt at vide om miljømæssige faktorer påvirker tolerancen overfor HDPer, og at vurdere om der er risiko for at der udvikles resistens når HDPer bruges udenfor deres naturlige miljø.

Formålet med dette Ph.D. studie var at undersøge hvordan to beslægtede Gram-positive patogene bakterier, *Listeria monocytogenes* og *Staphylococcus aureus*, reagerede på HDPer. Vi antog at stammer med et øget virulenspotentiale ville være mere tolerante overfor de antimikrobielle komponenter i det innate immunforsvar. Vi undersøgte en samling af 25 *L. monocytogenes* stammer og 16 *S. aureus* stammer der repræsenterede forskellige undergrupper, oprindelse og fænotypisk adfærd. Vi brugte fire modelpeptider, der repræsenterede de tre klasser af HDPer. Protamin er et lineært peptid der er rigt på prolin og arginin, plectasin er et defensin der er isoleret fra en svamp og novispirin G10 og dets derivat novicidin er lineære α-heliske peptider der også tilhører gruppen af cathelicidiner. Vi fandt ud af at stammer indenfor hver af de to arter af *L. monocytogenes* og *S. aureus* var lige sensitive overfor HDPer, og at denne sensitivitet ikke kunne beslægtes med forskelle i fænotypisk adfærd. Det vil sige at den potentielle brug af HDPer som alternative antibiotika ikke bliver forhindret af naturligt forekommende resistente bakterier. Det vides også at de miljømæssige forhold som en bakterie udsættes for forud for infektion kan påvirke den fysiologiske tilstand og dermed også evnen til at skabe infektion. Vi antog at ved at udsætte *L. monocytogenes* for stress faktorer som normalt findes i fødevarer (5% salt, pH 5,5 og 5°C)

ville der initieres et stressrespons i *L. monocytogenes* som ville resultere i en øget tolerance overfor HDPer. Vi observerede ikke nogen øget tolerance overfor HDPer efter hver af de tre stresspåvirkninger.

Endelig undersøgte vi en samling transposonmutanter i både *L. monocytogenes* og *S. aureus* for tilstedeværelsen plectasinresistente mutanter for at prøve om der kunne fremprovokeres resistens overfor HDPer ved en enkelt mutation. Vi identificerede en mutant i *S. aureus* med transposonet indsat i responsregulatoren *hssR* fra to-komponentsystemet HssRS. Denne mutant havde en to- til fire-fold øget tolerance overfor plectasin. Vi fandt ingen resistente mutanter i *L. monocytogenes*, hvilket antyder at udvikling af resistens gennem enkelte mutationer er forholdsvis usandsynlig.

For at opsummere har vi vist at der ikke forefindes naturligt tolerante mutanter i to populationer af hhv. *L. monocytogenes* og *S. aureus*, og at forudgående stresspåvirkninger ikke ændrede *L. monocytogenes* tolerance overfor en efterfølgende HDP-behandling. Desuden fandt vi at der ikke umiddelbart udvikles resistens gennem enkeltmutationer. Disse resultater understøtter at HDPer kan bruges som potentielle nye antibiotika.

L. monocytogenes' evne til at adaptere til specifikke omgivelser kan også føre til etablering af persisterende subpopulationer i forskellige naturlige nicher, f.eks. i fødevareprocesanlæg. Der er stor sandsynlighed for at sådanne persisterende stammer vil kontaminere fødevarer, og derfor er det i forbindelse med vurdering af fødevaresikkerheden vigtigt at undersøge disse stammers virulenspotentiale. Samlingen af *L. monocytogenes* stammer der er brugt i dette studie indeholder en persisterende subtype, RAPD type 9 (random amplified polymorphic DNA), som er blevet isoleret gentagende gange fra fiskeindustrien gennem en lang årrække. Det er tidligere blevet vist at disse stammer er lavvirulente i simple *in vitro* virulensmodeller og ikke-mammale modeller. I en mere kompleks biologisk model hvor der blev brugt gravide marsvin, viste det sig derimod at en af disse stammer inficerede placenta og fostre lige så effektivt som en højvirulent klinisk stamme. Vi antog at RAPD type 9 stammerne havde en øget evne til at gennemføre nogle af de specifikke trin der indgår i krydsningen af placenta-barrieren og at dette eventuelt kunne afspejles i sekvensforskelle i de virulensgener der er involveret i disse trin. Vi fandt at RAPD type 9 stammerne invaderede placentale trophoblastceller i et lavere niveau end andre kliniske stammer, og at dette sandsynligvis kan forklares af præmature stop codons i *inlA*. Ligeledes var RAPD type 9 stammernes celle-til-celle spredning i fibroblaster ikke anderledes end observeret for de kliniske stammer. Vi konkluderer at RAPD type 9 stammerne stadig kan antages at være lavvirulente i human listeriose, og at de ikke udgør en særlig risiko for gravide kvinder.

Contents

1. Introduction

To establish an infection successfully, pathogenic microorganisms must first overcome the barriers of the innate immune system. This first line of defense is composed of several cell types that utilize an array of effector mechanisms that all contribute to the physical and chemical defense lines. Epithelial cells line both the skin and mucosa and act as a physical barrier to prevent pathogens from entering. In addition, they secrete mucus and microbicidal compounds that serve as a chemical defense. Bacteria penetrating these barriers are met by phagocytic cells which engulf the pathogens and subsequently kill them intracellularly by the means of the same chemical compounds as the epithelial cells secrete. These chemical antimicrobial compounds can broadly be categorized into those mediating an oxygen-dependent killing and those mediating an oxygen-independent killing. The reactive oxygen species (ROS) such as hydrogen peroxide and superoxide that are produced in phagocytes constitute an important part of the first group. Antimicrobial peptides (AMPs) are one of the main contributors to the latter group (Flannagan et al. 2009).

Antimicrobial peptides (AMPs) constitute an evolutionary very well-conserved group of bacterial inactivator molecules and are widespread in the innate immune systems of mammals, insects, plants, and fungi (Zasloff 2002;Brogden et al. 2003). The fact that they have retained antimicrobial activity through millions of years suggests that development of resistance is relatively improbable. They are now often referred to as host defense peptides (HDP) as they besides the direct antimicrobial actions also have other immunomodulatory properties, like chemotactic activities that serve to attract and activate several effector cells of both the innate and adaptive immune system. HDPs thus enhance both the innate and adaptive immune responses thereby boosting infection-resolving immunity. Together this has prompted a massive interest in HDPs as novel antimicrobials as they can become an entirely new therapeutic approach to combat bacterial infections (Hancock and Sahl 2006).

Nevertheless, a cardinal feature of pathogenic bacteria is the evolution of advanced strategies to circumvent, resist or counteract the innate defense systems allowing the establishment of a temporary niche for bacterial survival at the epithelial cell surface from where the infection can be initialized. Such intrinsic resistance mechanisms are regulated by two-component systems that sense the host environment and regulate the expression of virulence or resistance genes accordingly (Mandin et al. 2005;Kraus et al. 2008). On the other hand, some host factors have been shown to induce down-regulation of virulence genes (Dorschner et al. 2006). Hence, a better understanding of the molecular basis of

bacterial resistance to HDPs will provide insights on how to design therapeutic HDPs that are not prone to provoke development of resistance.

This study focuses on two related gram-positive, pathogenic species, *Listeria monocytogenes* and *Staphylococcus aureus*, that represent different routes of infection. *L. monocytogenes* is a food borne pathogen entering the host via contaminated food products and gains access to the blood stream by invading the intestinal epithelium. *L. monocytogenes* disseminates in the blood and can cause general sepsis. From the blood stream it can cross both the blood-brain barrier giving rise to central nervous system infections such as meningitis and encephalitis as well as the placental barrier causing severe illness in the fetus leading to abortion, still birth or premature birth (Vazquez-Boland et al. 2001). *S. aureus* has a more complex disease pattern. It can, as *L. monocytogenes*, be a food borne pathogen causing food poisoning due to the production of enterotoxins. However, it is more often a community-acquired pathogen, gaining access to the host tissue through breaches in the skin and cause a variety of diseases ranging from superficial skin infections like boils and abscesses to more serious infections such as pneumonia, meningitis, septicemia, endocarditis, and osteomyelitis as well as toxic shock syndrome due to the production of Toxic Shock Syndrome Toxin 1 (TSST-1) (Lowy 1998).

Strains of both *L. monocytogenes* and *S. aureus* are not equally virulent (Roche et al. 2001;Buncic et al. 2001;Peacock et al. 2002;Melles et al. 2004) and also differ in their sensitivity to stresses encountered (Buncic et al. 2001;Lianou et al. 2006;Rode et al. 2007). Since HDPs and other components of the innate defense system may be viewed as stress factors, it can be hypothesized that differences in virulence between strains may reflect differences in their ability to circumvent and survive the stresses imposed by the innate immune system. The understanding of the natural variation in sensitivity of strains of pathogenic species to HDPs would be an essential part of evaluating the potential of HDPs in treatment. In addition, the environmental conditions to which a pathogen is exposed prior to infection can be decisive for its virulence potential. Adaptation to sub-lethal levels of environmental stress conditions induces a stress response in the bacteria that confers tolerance to lethal levels of stress and to other forms of stress as well, a phenomenon known as stress hardening (Lou and Yousef 1997). Thus, exposure to such environmental stresses may alter the physiological state of a pathogenic bacterium in a way that prime the pathogen for subsequent stages of infection (Garner et al. 2006b;Andersen et al. 2007;Werbrouck et al. 2009). Such an adaptation might also include tolerance to HDPs.

The ability to adapt to specific environmental conditions can also lead to persistence of bacteria in distinct ecological niches. The *L. monocytogenes* strain collection used in this study comprises a subset of persistent strains. These strains constitute a specific molecular subtype that is isolated repeatedly in the same processing plant, and even in the same places inside the plant during a period of several years (Rørvik et al. 1995;Norton et al. 2001;Vogel et al. 2001b;Wulff et al. 2006). Such strains are likely contaminants of food products and hence it is important to assess their virulence potential. Previous work have shown that a group of these persistent strains were low virulent in simple *in vitro* models and non-mammalian models (Jensen et al. 2008a) but in a more complex mammalian model, the pregnant guinea pig, a persistent strain infected the fetuses in the same level as a high-virulent clinical strain (Jensen et al. 2008b). This discrepancy between *in vitro* and *in vivo* assessment of virulence potential needs to be investigated further in order to understand the actual risk posed by such persistent bacteria. The occurrence of both sporadic cases as well as an outbreak of listeriosis caused by a persistent subtype of *L. monocytogenes* (Olsen et al. 2005b;Orsi et al. 2008) further emphasizes the need to investigate the virulence potential of such persistent strains.

Within the broader research perspective of understanding the infectious process in order to better design new antimicrobial therapeutics, the purpose of the study has been to investigate the response of different strains of *L. monocytogenes* and *S. aureus* to antimicrobial compounds of the innate immune defense. Specifically, the project has focused on AMPs or HDPs as these are potential novel antimicrobials. In addition, the study explored if the response to HDPs could be linked to the variation in the strains' phenotypic behavior, including expression of virulence-related factors and behavior in eukaryotic cell models.

As the innate immune system constitutes our first line of defense against invading pathogens, we hypothesized that any differences in virulence between strains of the same pathogenic species might be reflected in differences in tolerance to the stressors of the innate immune defense. Hence, we determined the natural variation in sensitivity of *L. monocytogenes* and *S. aureus* strains to HDPs and hydrogen peroxide (Gottlieb et al. 2008). Furthermore, the environmental stress conditions that pathogens are prone to be exposed to prior to infection might elicit a stress response that augments the infectious potential. We hypothesized that exposure to food-related environmental stress conditions could increase the tolerance of *L. monocytogenes* to subsequent HDP treatment (Gottlieb and Gram 2009). Also, to examine the potential development of resistance to HDPs outside the natural

environment, we investigated if tolerance to HDPs could be induced by transposon mutagenesis (Thomsen et al. 2009).

To further investigate the differences in virulence potential between *L. monocytogenes* strains, we compared the behavior of the subgroup of persistent strains to clinical strains in eukaryotic cell models and determined if differences in phenotypic behavior could be explained by systematic differences in virulence gene sequences. We hypothesized that the otherwise low-virulent, persistent strains had a predilection for the placenta and hence had a specific ability to execute one (or several) of the steps involved in transmission of *L. monocytogenes* across the placenta membrane (Holch et al. 2009).

The thesis consists of an overview section and four papers. The overview section gives an introduction to the innate immune defense and the current knowledge of HDPs and their antimicrobial and immunomodulatory functions. In this context the potential use of HDPs as novel antimicrobials is discussed. In addition the two pathogenic bacteria *L. monocytogenes* and *S. aureus* are introduced and various phenotypic and genotypic analyses as well as eukaryotic cell models for assessment of their virulence potential is discussed. The experimental work and the results obtained during the Ph.D. project are described in four papers.

2. Innate immune defense against bacteria

The myriads of microorganisms that an individual faces every day only rarely cause disease due to the innate immune system that acts as a first line of defense. Pathogenic microorganisms are prevented from entering by physical barrier properties, or are combated through the action of antimicrobial compounds. This chapter focuses on the antimicrobial compounds of the innate immune system, in particular the host defense peptides. A general introduction to the actions of the innate immune system and the induction of the adaptive immune response is given in order to understand the immunomodulatory functions of HDPs.

2.1. The first line of defense

To establish an infection in a host organism, pathogens must first overcome the barriers of the innate immune system. These barriers are composed of several cell types that utilize an array of effector mechanisms that all contribute to the physical and chemical defense lines (Figure 1).

Epithelial cells line both the skin and mucosa and act as a physical barrier preventing pathogens from entering. In addition, they secrete mucus and microbicidal compounds that serve as a chemical defense. Bacteria penetrating these barriers are met by phagocytic cells which engulf the pathogens and subsequently kill them intracellularly. The phagocytic cells kill the pathogens by the means of the same chemical compounds as are secreted by the epithelial cells.

Differences in virulence between strains of pathogenic bacteria might be caused by differences in the ability to overcome or circumvent the stresses imposed by the innate immune system.

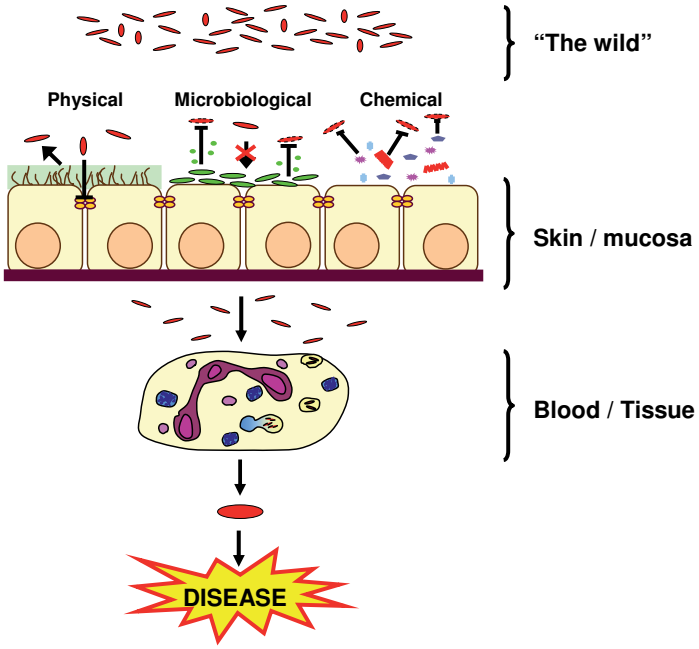


Figure 2.1: The barriers of the innate immune system. The mechanical barrier function of the epithelial cells consists of tight junctions and mucus. The normal flora of nonpathogenic bacteria on both the skin and gastrointestinal mucosa compete with pathogenic bacteria for nutrients and attachments sites and may even secrete antimicrobial substances. The chemical barrier consists of HDPs and compounds that generate an oxidative burst, as well as secreted fatty acids and various enzymes such as lysozyme. If the epithelial barrier is breached, the phagocytic cells in the host tissues contain antimicrobial compounds in various granules. These include HDPs, lysozyme, acid hydrolase, and myeloperoxidase that generates an oxidative burst. NADPH oxidase in the cytosol is also responsible for generating an oxidative burst. © Gottlieb 2007.

2.1.1. Epithelial cells

Epithelial cells line all internal and external surfaces of the body, including the skin and the gastrointestinal and respiratory mucosa. They are held together by tight junctions that effectively form a physical barrier. In addition, they secrete mucus, a viscous fluid that contains many glycoproteins called mucins. Mucus can coat microorganisms, thus preventing their adherence to the epithelium and aid in expelling the microorganism. This is seen in the flow of mucus driven by the cilia in the respiratory epithelium or the peristaltic movement in the gastrointestinal tract (Acheson and Luccioli 2004). The importance of

mucus in immunity is seen for example in cystic fibrosis patients, where failure in mucus production leads to frequent lung infections.

The epithelial cells produce several chemical antimicrobial compounds that can broadly be categorized into those mediating an oxygen-dependent killing and those mediating an oxygen-independent killing. Compounds that generate an oxidative burst constitute the first group, whereas the antimicrobial peptides (AMPs) or host defense peptides (HDPs) constitute the majority of the non-oxygen dependent compounds. HDPs are either constitutively expressed at sites where initial interaction with potential invading microorganisms occurs, or are induced upon recognition of injury. In addition, proteolytic enzymes like lysozyme are secreted in saliva, sweat and tears, pepsin is secreted in the gut, and the skin produces fatty acids that also have antimicrobial effect.

2.1.2. Phagocytes

Pathogens that succeed in crossing the epithelial surfaces are removed by phagocytes in the underlying tissues. Macrophages are derived from blood-circulating monocytes and reside in tissues. They are found in large numbers in connective tissues in association with the gastrointestinal tract, the respiratory tract, and along certain blood vessels in the liver (where they are known as Kupffer cells). Macrophages are complemented by the recruitment of large numbers of neutrophils to the site of infection. Neutrophils or polymorphonuclear neutrophilic leukocytes (PMNs) is the second large family of phagocytic cells and are circulating in the blood. They are not present in normal healthy tissues but are recruited to the site of infection by HDPs and cytokines and chemokines that are secreted as a result of tissue injury.

The interaction between phagocytes and pathogens can be either direct - through recognition of pathogen-associated molecules such as surface carbohydrates, peptidoglycans or lipoproteins by pattern recognition receptors - or indirect by opsonins. Upon engulfment of pathogens, phagocytes undergo a drastic maturation to acquire the microbicidal and degradative features associated with innate immunity. These include the progressive acidification of the phagosome as well as the acquisition of HDPs and hydrolases (Flannagan et al. 2009).

The internalization and subsequent destruction of pathogens in phagocytes are key features of the innate immune response that promote antigen presentation and the activation of adaptive immunity.

2.1.3. Activation of adaptive immunity

If (or when) microbial pathogens pass the first line of defense of the innate immune system, the adaptive immune system is activated: Macrophages and dendritic cells engulf the pathogens and present pathogen-derived antigens on their surfaces. When these antigen-presenting cells encounter naïve, circulating T-cells the T-cells are activated and proliferate to generate a large number of antigen-specific effector T-cells, of which there are three different kinds. The relative production of each subset of effector T-cells depends on the nature of the infection. Intracellular pathogens such as vira and *L. monocytogenes* are eliminated by cytotoxic CD8 T-cells that migrate to the infected tissue and kill the infected host cells. Other pathogens are killed by the two kinds of helper CD4 T-cells. Intravesicular pathogens such as *Mycobacterium tuberculosis* are eliminated via CD4 T_H1 cells that activate the infected macrophages. Extracellular pathogens (i.e. most bacteria) are eliminated by CD4 T_H1 and T_H2 cells by induction of engulfment by macrophages and activation of specific B-cells to produce antibodies, respectively.

2.2. Antimicrobial compounds of the innate immune defense

As mentioned, the antimicrobial compounds of the innate defense can broadly be divided into those mediating an oxygen-dependent killing and those that mediate a non-oxygen-dependent killing.

2.2.1. Oxygen-dependent

The generation of reactive oxygen species (ROS) in professional phagocytes is catalysed by NOX2 NADPH oxidase that transfers electrons from cytosolic NADPH to molecular oxygen, releasing O_2^- into the phagosomal lumen (Quinn and Gauss 2004). ROS production is most prominent in neutrophils. In the phagosomal lumen, O_2^- can dismutate to H_2O_2, which can in turn react with O_2^- to generate hydroxyl radicals and singlet oxygen (Minakami and Sumimoto 2006). H_2O_2 can also be converted by myeloperoxidase into hypochlorous acid and chloramines (Flannagan et al. 2009).

In addition to ROS, nitric oxide ($NO^\bullet$) and the reactive nitrogen species (RNS) derived from it are important antimicrobial effectors. RNS are primarily produced in macrophages by the inducible nitric oxide synthase, NOS2, isoform. RNS production requires de novo synthesis of NOS2 in response to proinflammatory agonists (Fang 2004). $NO^\bullet$ is synthesized on the cytoplasmic side of phagosomes, and diffuse across membranes to reach intraphagosomal targets. In the luminal environment, where it encounters ROS, $NO^\bullet$ can undergo either

spontaneous or catalytic conversion to a range of RNS, including nitrogen dioxide ($NO_2^{\bullet}$), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and nitroxyl (HNO) (Fang 2004).

ROS and RNS synergize to exert highly toxic effects on intraphagosomal microorganisms. They interact with numerous microbial targets, such as thiols, metal centers, protein tyrosine residues, nucleic acids and lipids. As a result, proteins are inactivated and lipids are converted by oxidative damage. In addition, microbial DNA can undergo irreparable damage. Together, these reactions can impair bacterial metabolism and ultimately inhibit replication (Imlay 2003).

However, many pathogens can survive the oxidative burst, for example by secreting enzymes such as catalase that neutralizes ROS/RNS. In the present study, we have tested if strains of *L. monocytogenes* and *S. aureus* that presumably represent different levels of virulence as discussed in chapters 4 and 5, varied in their tolerance to H_2O_2 as a model compound of the oxidative burst mediated by the innate immune defense. We found no difference in sensitivity in a collection of 25 *L. monocytogenes* and 16 *S. aureus* strains (see Table 3.1, chapter 3) (Gottlieb et al. 2008).

2.2.2. Non-oxygen-dependent compounds

Non-oxygen-dependent compounds comprise both large and small antimicrobial proteins and peptides. The large proteins are often lytic enzymes, such as lysozyme, nutrient-binding proteins or hydrolases, and proteases that target bacterial carbohydrates, lipids, and proteins (Flannagan et al. 2009). Lactoferrin is an example of a nutrient-binding protein. It is contained in neutrophil granules and released into the phagosomal lumen, where it sequesters iron that is required by some bacteria (Masson et al. 1969). In addition, the phagosome is acidified due to the action of the V-ATPases causing a luminal pH as low as 4.5 (Desjardins et al. 1994;Huynh and Grinstein 2007;Flannagan et al. 2009). The acidification of the phagosome serves two purposes. First, to impair the metabolism of pathogens, and second, to favor the activity of many host hydrolytic enzymes that have acidic pH optima. In addition, the transmembrane H^+-gradient generated by V-ATPases both causes extrusion of essential microbial nutrients from the phagosomal lumen and facilitates the generation of superoxide (O_2^-) due to the surplus of H^+ that counteracts the negative charges translocated by the NADPH oxidase (Flannagan et al. 2009).

The smaller antimicrobial peptides, which are the focus of this thesis, are a diverse group of peptides that have an essential role in the innate immune defense of all living organisms. They are small, ribosomally-synthesized peptides, most in the range of 10-50 amino acids.

They have a positive net charge and an amphiphatic structure (Zasloff 2002;Brogden et al. 2003;Marshall and Arenas 2003;Powers and Hancock 2003;Brogden 2005). The antimicrobial peptides exhibit such great diversity that they can only be categorized broadly into three classes on the basis of their secondary structure (Table 2.1).

Table 2.1: Classification of host defense peptides based on secondary structure and examples of HDPs belonging to each class. Most organisms express multiple peptides from several of these structural classes. Underlined HDPs are used in this study. Reviewed in (Zasloff 2002;Brogden et al. 2003;Marshall and Arenas 2003;Powers and Hancock 2003;Brogden 2005).

Peptide	Origin
Linear peptides forming helical structures	
hCAP-18/LL-37	Human neutrophil granules Human epithelial cells (skin, lung, gut, mammary gland and epididymis)
Novispirin G10	Synthetic (from ovispirin-1 from SMAP-29)
Novicidin	Derivate of novispirin G10
Cecropins	Insect hemolymph
Margainins	Frog skin
Pleurocidin	Fish skin mucous
Buforin II	Human gastric mucosa
Cysteine-stabilised peptides with a β-sheet	
Human defensins	
HNP1-4	Neutrophils
HD5-6	Paneth cells in the small intestine
HBD1-4	Epithelial cells (gastrointestinal and respiratory tract, skin)
Plectasin	Fungus
Protegrins	Pig leukocytes
Tachyplesins	Horseshoe crab
Penaeidins	Shrimp
Linear peptides rich in specific amino acids (Pro, Gly, His, Trp)	
Protamine (Pro, Arg)	Fish spermatozoa
Histatins (His)	Human (primate) saliva
Bactenins (Pro, Arg)	Bovine neutrophils
PR-39 (Pro, Arg)	Pig small intestine
Indolicidin (Trp, Pro)	Bovine neutrophils

Across these three classes are a group of peptides called cathelicidins. These are peptides that differ greatly in their sequences, structures and sizes but share a highly conserved N-

terminal structural domain (cathelin) that is linked to a C-terminal peptide possessing the antimicrobial activity.

In humans and other mammals, the two main HDP families are the defensins and the cathelicidins (Zasloff 2002;Brogden et al. 2003). The sole human cathelicidin is synthesised as a preproprotein named human cationic protein 18 kDa (hCAP18) whose 37 C-terminal amino acids constitute the active peptide, LL-37. LL-37 is generated by proteolytic processing of hCAP18 by various tissue-specific proteinases (Eckmann and Kagnoff 2005). hCAP18 is synthesised and stored in secondary neutrophilic granules but has also been found in various epithelial sites, mast cells and a subpopulation of monocytes and lymphocytes (Ganz 2003).

The human defensins have three pairs of disulfide bonds and a β-sheet structure. They are sub-divided into six α-defensins and four β-defensins based on the arrangement and spacing of the three disulfide bonds, as shown in Figure 2.2.

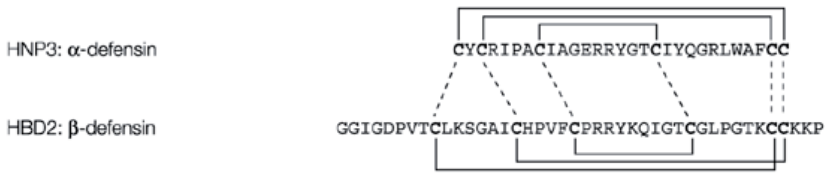


Figure 2.2: Sequence and disulphide bonds in a human α- and β-defensin. The disulphide bonds are indicated by solid lines and the corresponding cysteines in α- and β-defensins by dotted lines. From (Ganz 2003).

The α-defensins human neutrophil peptides 1-4 (HNP1-4) are expressed in neutrophils whereas human defensin 5 and 6 (HD-5 and HD-6) are expressed predominantly in Paneth cells in the small intestine. The four human β-defensins, HBD1-4, are expressed in epithelial cells such as those lining the intestinal and respiratory tracts and the skin, and are thus important in protection from environmental pathogens (Ganz 2003).

In leukocytes and Paneth cells the defensins are stored in granules and released for antimicrobial action into phagocytic vacuoles and intestinal crypts, respectively, and the physiological concentrations of HDPs can be as high as > 10 mg/mL. In the various secretory

and barrier epithelial cells the average concentration reaches 10-100 µg/mL. *In vitro* antimicrobial activity is observed to be as low as 1-10 µg/mL (Ganz 2003).

Bacteria also produce ribosomally synthesized antimicrobial peptides, collectively named bacteriocins. These include nisin that is widely used as a food preservative, as well as pediocin PA-1 and lacticin 3147 (Cleveland et al. 2001). Bacteria also produce peptide antimicrobials using large multifunctional enzymes known as non-ribosomal-peptide synthetases. Prominent examples of these include the polymyxins and gramicidins (Hancock and Sahl 2006).

2.3. Host defense peptides - mechanisms of action

HDPs have several mechanisms of action. They have direct antimicrobial actions, and due to their cationic, amphiphilic nature, HDPs have been suggested to be “dirty drugs”. They target many different microbial targets at the same time but with modest potency instead of blocking a specific high-affinity target, thereby making development of resistance difficult (Peschel and Sahl 2006). In addition, they have immunomodulatory actions, boosting both innate and adaptive immune responses which favor resolution of infection, hence the name host defense peptides rather than just antimicrobial peptides.

2.3.1. Direct antimicrobial action: membrane-acting HDPs

The wide spectrum of activity and the speed of action (often within minutes *in vitro*) of most HDPs indicate that they target the bacterial membrane. The Shai-Matsuziaki-Huang model (SMH-model) describes the activity of most HDPs (Zasloff 2002). The model proposes that the peptides interact with the membrane via peptide-lipid interactions rather than receptor-mediated recognition processes. The fact that synthetic all-D amino acid enantiomers of the peptides exhibit the same antimicrobial spectra as their all-L native counterparts indicates that the antimicrobial activity does not involve stereo-specific protein receptors.

In general, the cationic HDPs are attracted by electrostatic forces to the anionic phospholipids and phosphate groups on lipopolysaccharide (LPS) on Gram-negative bacteria, and teichoic acids on Gram-positives (Shai 1999). The peptides have low affinity for the outer leaflet of plant and animal membranes, which are composed of lipids with no net charge (Zasloff 2002). In addition, the presence of membrane-stabilising cholesterol has been shown to further protect eukaryotic cells against HDPs (Matsuzaki 1999). After the initial attraction, HDPs must traverse capsular polysaccharides (and teichoic and lipoteichoic acid in Gram-positives) in order to reach the cytoplasmic membrane and interact with the

lipid bilayers (Brogden 2005). Hydrophobic interactions are the driving force of the following permeabilisation and/or disruption of the membrane. When associated with the membrane the peptides exhibit two distinctly different physical states of binding. At low peptide-to-lipid ratios the peptides are embedded in the lipid headgroup region parallel to the peptide-lipid interface in a functionally inactive state. The embedment stretches the area of the membrane and results in membrane thinning. As the peptide concentration increases, a certain threshold level is reached, and the peptides shift from the parallel orientation to a perpendicular orientation leading to insertion into the cytoplasmic membrane and the formation of multiple pores. The transition occurs when the energy levels of the surface-state and the integrated state are equal. The susceptibility of a cell to HDP depends on this threshold level that is determined by the lipid composition of the cell membrane (Huang 2000).

Four models have been proposed to describe the membrane permeabilisation. The four models - the aggregate model, the toroidal-pore or wormhole model, the barrel-stave model, and the carpet model - are illustrated in Figure 2.3. In certain cases the peptide enters into the interior of the target cell via these pores, presumably to exert additional antimicrobial activities.

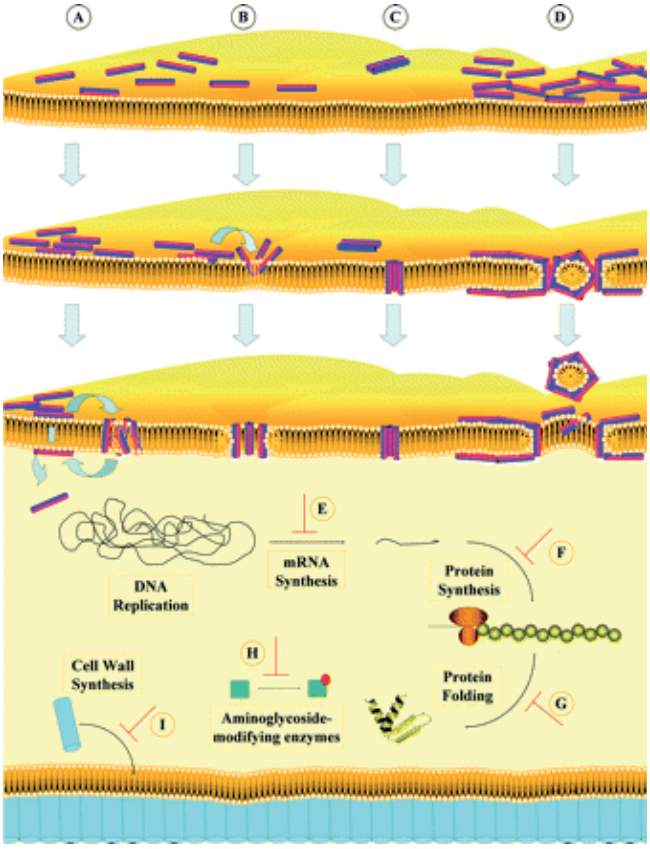


Figure 2.3: Mechanism of action of host defense peptides. The bacterial membrane is shown as a yellow lipid bilayer and the peptides are shown as cylinders with the hydrophilic regions in red and the hydrophobic regions in blue. (A-D) explain mechanisms of membrane permeabilization. (A) The aggregate model. (B) The toroidal-pore ("wormhole") model: Peptide helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore to connect the two leaflets of the membrane. The toroidal model differs from the barrel-stave model as the peptides are always associated with the lipid head groups even when they are vertically inserted in the lipid bilayer. (C) The barrel-stave model: Peptide helices form a bundle in the membrane and the hydrophobic peptide regions align with the membrane lipid core region leading to transmembrane pore-formation. (D) The carpet model: Peptides accumulate on the surface of the bilayer forming a peptide-carpet that is thought to disrupt the lipid bilayer in a detergent-like manner leading to the formation of micelles. (E-I) explain mechanism of action of non-membrane acting HDPs. Adapted from (Jenssen et al. 2006).

Amphiphatic α-helical HDPs permeate membranes predominantly via the “carpet” mechanism (Shai 1999). Defensins are also thought to permeate the membranes via the carpet or toroidal pore mechanism although the specific arrangement of defensin molecules in the pores remains unknown (Ganz 2003).

Pore formation (as in formation of any ion- or water-permeable structure in the membrane) seems to be a central point in the peptide-membrane interaction. The pores formed by various HDPs vary in size but are all ≥ 20 Å, thus allowing the passage of ions and small molecules. This would lower the proton gradient and destroy the membrane potential, stopping ATP production and all cellular metabolism, leading to cell death (Huang 2000). These pores can be either stable as revealed by release of intracellular components in an “all-or-nothing” manner, or non-stable as shown by a partial release of intracellular markers. This is probably due to destabilization of the pores by electrostatic repulsions between highly cationic HDPs (Ganz 2003).

The formation of pores or general disruption of the membrane can be experimentally demonstrated by measuring the leakage of intracellular components to the extracellular milieu. We have determined the leakage of ATP upon treatment with three different peptides, representing the three different classes (Figure 2.4). Protamine caused leakage of ATP to the extracellular environment as also shown by (Johansen et al. 1997), suggesting that it acts on the membrane. Microscopic examination of cell cultures exposed to protamine suggested that it not only formed small pores but disrupted the membrane in a detergent-like manner (Figure 2.4c). This is supported by Johansen et al. (1997) that also showed that protamine caused leakage of high-molecular-weight compounds such as β-galactosidase. Plectasin did not appear to act on the membrane on *S. aureus* as no ATP leakage was seen. Plectasin treatment of *L. monocytogenes* did cause a small leakage of ATP although not to the same extent as seen with protamine. This leakage is probably due to secondary effects as the slow killing kinetics observed for plectasin suggest that is not acting on the membrane (Mygind et al. 2005). Novicidin also resulted in leakage of ATP and could thus act on the membrane. However, the extremely rapid action as well as the fact that no ATP was left 5 minutes after start of exposure suggest that novicidin could have additionally intracellular actions, such as activation of ATPases. Cell-free experiments where a standard ATP solution was treated with novicidin did not result in decreasing ATP levels (data not shown), suggesting that novicidin is not directly degrading ATP.

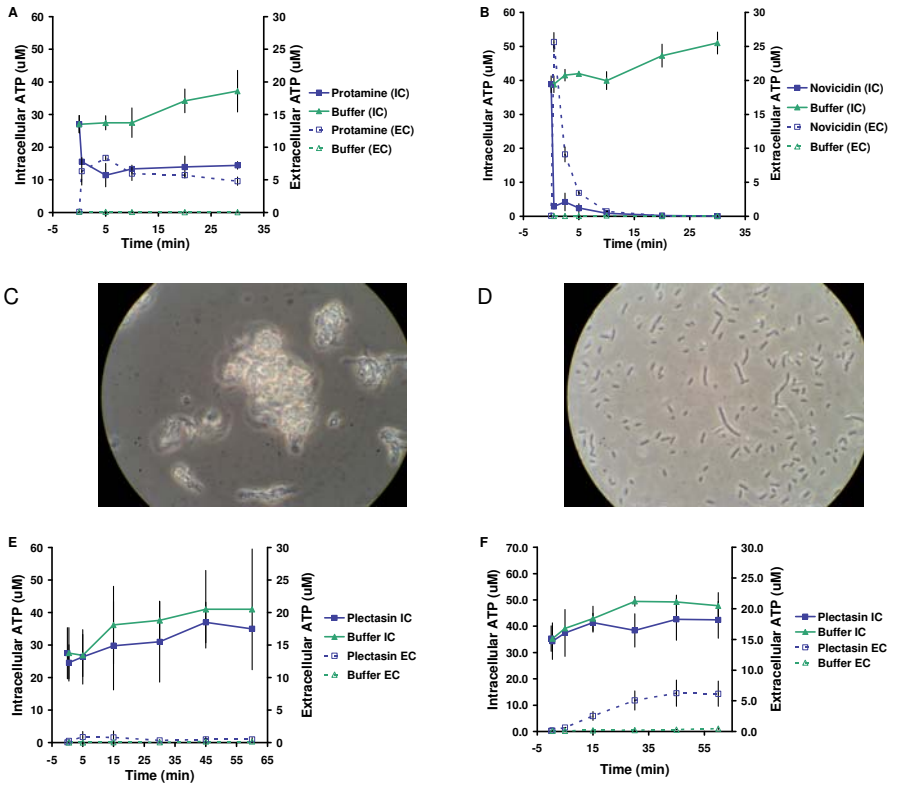


Figure 2.4: Measurement of ATP leakage after treatment with HDPs. (A) ATP leakage from *S. aureus* after treatment with protamine. (B) ATP leakage from *S. aureus* after treatment with novicidin. (C) Photograph of *L. monocytogenes* after treatment with protamine (1,000x magnification). (D) Photograph of *L. monocytogenes* after treatment with plectasin (1,000x magnification). (E) ATP leakage from *S. aureus* after treatment with plectasin. (F) ATP leakage from *L. monocytogenes* after treatment with plectasin. Adapted from (Thomsen et al. 2009).

Together, the three different leakage profiles observed after treatment with the three peptides suggest that they have different mechanisms of action. These results are physiologically meaningful, since the simultaneous use of host defense molecules with different actions will make the defense against microorganisms more effective and reduce the risk of development of resistance as is discussed in chapter 3.

2.3.2. Direct antimicrobial action: Non-membrane acting HDPs

Increasing evidence indicates that HDPs have additional antimicrobial actions besides the pore-forming or general membrane disrupting actions described above. These can be non-membrane external targets that can be activated by HDPs to induce lysis of the bacteria. Examples include the activation of bacterial autolysins that is normally inhibited by the presence of cell wall components like lipoteichoic and teichuronic acids (Bierbaum and Sahl 1987) and enhancement of the activity of host-derived secretory phospholipase, thus showing a synergistic effect of HDPs and other host defense factors (Zhao and Kinnunen 2003). HDPs have been shown to interact with periplasmic and cytoplasmic macromolecules once they have penetrated the cell wall (Figure 2.2 and Table 2.2). The mechanism of action of a particular HDP most likely varies according to the bacterial target cell, the concentration at which the HDP is assayed, and the physical properties of the interacting membrane (Jenssen et al. 2006). Many HDPs have been attributed potentially false membrane-targeting actions because the mechanism have been investigated using concentrations high above MIC values, exceeding the MIC-equivalent peptide:lipid ratio, or masking any potential intracellular effects (Shai 1995;Patrzykat et al. 2002). Such an effect could account for the ATP leakage seen after plectasin-treatment of *L. monocytogenes*.

In order to access their intracellular targets, HDPs must cross the bacterial membrane without causing permeabilisation. Arginine-rich peptides readily translocate across both cellular and nuclear membranes (Futaki et al. 2001) by endocytosis (Richard et al. 2003) or pinocytosis (Wadia et al. 2004). Protamine used in this study is a linear peptide rich in proline and arginine, hence, in lower concentrations it might cross cell membranes and have additionally intracellular targets.

Buforin II accumulates in the cytoplasm where it inhibits cellular functions by binding to DNA and RNA (Park et al. 1998). We have examined the DNA-binding properties of plectasin and another plectasin-like defensin, eurocin, using a gel retardation assay as described by (Park et al. 1998). We found that these peptides do not bind to DNA (Figure 2.5) (Thomsen et al. 2009).

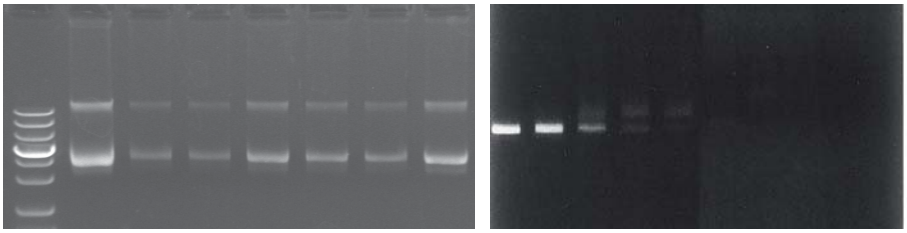


Figure 2.5: Gel retardation assay examining the DNA binding properties of HDPs. (A) Plectasin (0, 2.5, 5, 10, 20, 40 or 80 µg/ml) was incubated with 100 ng of plasmid DNA for 1 hour at room temperature and applied to a 1% agarose gel electrophoresis. The migration of plasmid DNA was not inhibited, indicating that plectasin does not bind DNA. Data not shown from (Thomsen et al. 2009). (B) A comparable experiment using buforin II that is known to bind DNA. Once a certain threshold limit of peptide:DNA ratio is reached the migration of DNA is inhibited, indicating that the peptide bind DNA. From (Park et al. 1998).

However, the gel retardation assay is an indirect, *in vitro* determination of DNA binding/inhibiting activity. By measuring *in vivo* incorporation of [^{3}H]thymidine or [^{3}H]uridine into DNA and RNA, respectively, one can determine if HDPs directly inhibit DNA and RNA synthesis. Inhibition of nucleic acid synthesis has been demonstrated for HDPs from different structural classes, both the α-helical peptides such as derivates of pleurocidin and dermaseptin (Patrzykat et al. 2002), the β-sheet, cysteine stabilized peptides such as the human HNP-1 (Lehrer et al. 1989), and the linear, Trp- and Pro-rich bovine peptide, indolicidin (Subbalakshmi and Sitaram 1998). Likewise, analyzing the incorporation of radioactively marked amino acids can determine if a HDP inhibits protein synthesis, such as shown for PR-39 (Boman et al. 1993) and derivates of pleurocidin and dermaseptin (Patrzykat et al. 2002). Intracellular enzymatic activity can also be the target for pyrrhocidin that specifically inhibits the ATPase activity of DnaK, thus preventing its chaperone activity leading to accumulation of misfolded proteins in the cell cytoplasm and death (Otvos et al. 2000).

Two bacterial antimicrobial peptides, the lantibiotics nisin and mersacidin, both interfere with the cell wall precursor lipid II, thus preventing peptidoglycan formation and inhibiting cell wall synthesis (Hechard and Sahl 2002).

Table 2.2: Summary of HDP antimicrobial mechanisms of action (MOA).

MOA	Examples of HDPs	Reference
Membrane-acting		
Aggregate	Indolicidin	(Hancock and Chapple 1999;Wu et al. 1999)
Toroidal pore	LL-37	(Henzler Wildman et al. 2003)
	Magainin 2	(Matsuzaki et al. 1996;Yang et al. 1998)
	Protegrin-1	(Yamaguchi et al. 2002)
	Melittin	(Yang et al. 2001b;Lee et al. 2004)
Barrel-stave	Alamethicin	(Brogden 2005)
Carpet	Cecropin	(Gazit et al. 1995)
	Ovispirin	(Yamaguchi et al. 2001)
	Melittin	(Naito et al. 2000)
Non-membrane acting		
Nucleic acid synthesis	HNP-1, HNP-2	(Lehrer et al. 1989)
	Buforin II	(Park et al. 1998)
	Indolicidin	(Subbalakshmi and Sitaram 1998)
	Pleurocidin	(Patrzykat et al. 2002)
	Dermaseptin	(Patrzykat et al. 2002)
	PR-39	(Boman et al. 1993)
	Tachyplesin II	(Yonezawa et al. 2002)
Protein synthesis	HNP-1, HNP-2	(Lehrer et al. 1989)
	Pleurocidin, dermaseptin	(Patrzykat et al. 2002)
	PR-39	(Boman et al. 1993)
Protein folding	Pyrrhocoricin	(Otvos et al. 2000)
Cell wall (lipid II)	Bacteriocins (nisin, mersacidin)	(Hechard and Sahl 2002)

In summary, the cationic antimicrobial peptides have been proposed to have a multitarget mechanism of action, interacting with multiple anionic targets of the bacterial cell, including negatively charged lipids at the membrane, nucleic acids and intracellular enzymes (Powers and Hancock 2003).

2.3.3. Immunomodulatory actions

In addition to their direct antimicrobial action, HDPs have been demonstrated to confer protection against infection by indirect mechanisms as well. The immunomodulatory actions that both function to favor the resolution of infection and reverse potentially harmful effects of

inflammation (Figure 2.6) have eventually lead to a change of names from the more narrow antimicrobial peptides (AMP) to host defense peptides (HDP).

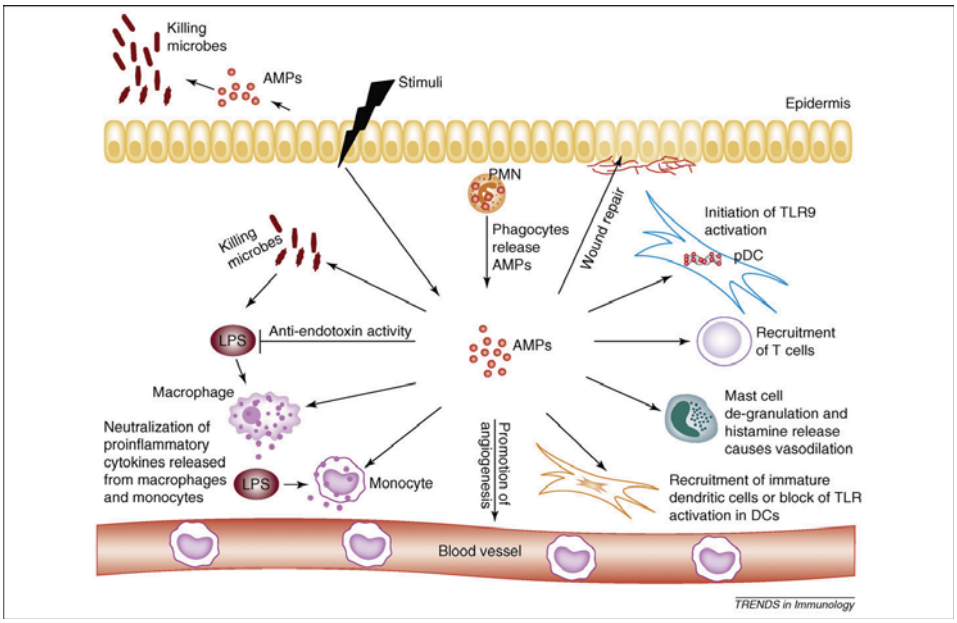


Figure 2.6: The multiple functions of HDPs in host defense. Apart from the direct antimicrobial actions, HDPs have immunomodulatory functions. HDPs have chemotactic activity that either directly or by stimulating secretion of chemokines and cytokines serves to attract and activate several cell types of both the innate and adaptive immunity. In addition HDPs regulate inflammation by inhibiting bacterial endotoxins and neutralize pro-inflammatory cytokines and promote wound healing and angiogenesis. From (Lai and Gallo 2009).

HDPs act directly or induce expression of chemokines and cytokines to recruit leukocytes to the site of infection. Both α-defensins HNP-1 and HNP-2 (Territo et al. 1989), β-defensins HBD-2, HBD-3, and HBD-4 (Niyonsaba et al. 2002;Yang et al. 2002), and LL-37 (Yang et al. 2000) have been shown to directly recruit monocytes and macrophages to the site of inflammation, thereby enhancing the initial phagocytosis and clearing of invading pathogens. In addition, both α-defensins (Yang et al. 2001a), β-defensins (Yang et al. 1999), and LL-37 (Yang et al. 2000) have induced recruitment of dendritic cells and T-cells to sites of infection, thus promoting the cellular immune response by facilitating antigen-presentation to naïve, circulating T-cells. HDPs also induce the secretion of chemokines and cytokines from both epithelial cells (Niyonsaba et al. 2005;Niyonsaba et al. 2007) and phagocytic cells (Funderburg et al. 2007;Yu et al. 2007).

Another important immunomodulatory role is the regulation of Toll-like receptor (TLR)-dependent inflammatory responses. Normally, inflammation serves to deliver additional effector cells and molecules to the site of infection as well as containing the infection locally. Bacterial products such as LPS stimulate inflammation via TLR-4. Uncontrolled inflammation causes tissue damage and can eventually lead to sepsis. Cathelicidins have been shown to inhibit TLR-4-mediated induction of cytokine release (Di Nardo et al. 2007;Morioka et al. 2008), and LL-37 can selectively inhibit TNF-α release from human monocytes and macrophages stimulated by LPS and LTA (Mookherjee et al. 2006). In addition, many HDPs can bind and neutralize LPS, thus providing an alternative mechanism of interfering with LPS response (Chen et al. 2006;Rosenfeld et al. 2006).

Several HDPs have also been shown to promote wound healing, serving to limit the infection by closing the site of entry. Cathelicidins stimulate the migration of keratinocytes required for re-epithelialization of the wound (Gallo et al. 1994;Tokumaru et al. 2005), HBD-2 and LL-37 stimulates migration, proliferation and formation of vessel-like structures of endothelial cells, thus promoting angiogenesis important for wound neovascularization (Koczulla et al. 2003), and LL-37 have also been shown to have anti-fibrotic effects that might benefit normal wound repair (Park et al. 2009).

In summary, HDPs have immunomodulatory functions that boost both the innate and adaptive responses to infection, dampen the potential tissue damage due to inflammation, and finally promote wound healing and tissue regeneration that function to close the site of entry of the pathogen, hence favoring the resolution of infection.

2.4. Bacterial evasion of innate immune mechanisms – intrinsic resistance to HDPs

The ability to survive the antimicrobial actions of HDPs due to intrinsic resistance mechanisms is an essential virulence property to many microbial pathogens as it allows them to establish a niche on the host surface from where the infection can be initiated (Miller et al. 2005). Here the various strategies employed by pathogenic bacteria to circumvent the actions of HDPs are presented.

Microorganisms use a number of resistance strategies to circumvent HDP-mediated killing (Figure 2.7). These can largely be categorised as mechanisms that alter the bacterial cell wall or membrane, inhibit HDP action (either by HDP binding/inactivation or by HDP degradation), downregulate HDP expression, or actively transport HDPs by energy-dependent influx/efflux systems.

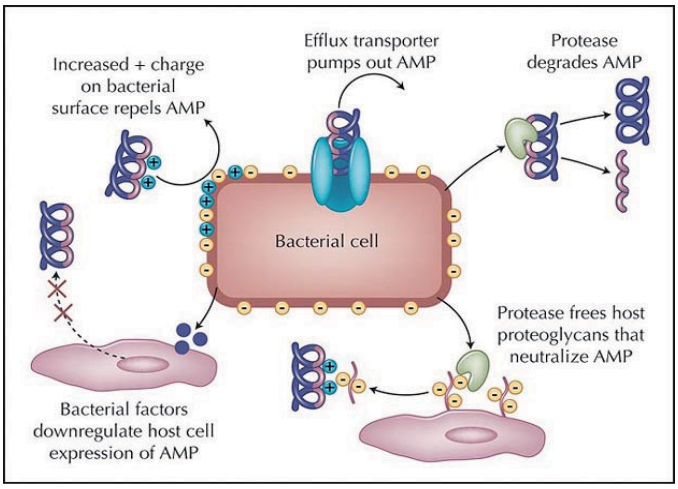


Figure 2.7: Bacterial mechanisms of HDP resistance. From (Gallo and Nizet 2003).

S. *aureus* alters the membrane composition via the *dlt* operon. This operon encodes proteins that transport D-alanine to the surface teichoic acids and catalyse the esterification of the phosphate groups of the teichoic backbone with the positively charged D-alanine. This causes a reduction of the net negative charge and thereby reduced binding of cationic HDPs (Peschel et al. 1999). Likewise, the staphylococcal *mprF* is involved in the biosynthesis of lysylphosphatidylglycerol (LPG) by catalysing the formation of the basic phospholipid by transfer of L-lysine from lysyl-tRNA to phosphatidylglycerol thus decreasing the attraction and binding of cationic HDPs by the bacteria (Peschel et al. 2001). Inactivation of the *dlt* operon or *mprF* resulted in increased sensitivity towards human HNP1-3, porcine protegrins and tachyplesins (Peschel et al. 1999;Peschel et al. 2001). We have shown that these two strains also exhibited increased sensitivity to plectasin, novicidin and novispirin G10 and to a lesser extent also protamine (Table 3.1) (Gottlieb et al. 2008). Interestingly, wild-type strains bearing additional copies of the dlt operon produced teichoic acids with higher amounts of D-alanine esters and were consequently less sensitive to antimicrobial peptides (Peschel et al. 1999). This suggests that strain differences in virulence can be caused by different expression levels of such intrinsic resistance genes. Homologues of these two staphylococcal genes have been found in many other bacteria, including *L. monocytogenes* (Abachin et al. 2002;Thedieck et al. 2006) and *Streptococcus pneumoniae* (Kovacs et al. 2006). Others have identified the staphylococcal IsdA surface protein that is produced in response to host factors (iron deprivation) and actually increases the negative charge of the membrane. However, it also makes the membrane more hydrophilic which renders *S. aureus*

more resistant to both HDPs and skin fatty acids that require hydrophobic interaction with the membrane for their activity (Clarke et al. 2007).

In Salmonellae and other Gram-negative bacteria the PhoPQ two-component regulatory system controls the expression of genes that are involved in the alteration of the fluidity of the outer membrane due to increased hydrophobic interactions between Lipid A acyl tails. In *Salmonella enterica* Typhimurium this is accomplished by the action of *pagP* (Pho-P activated gene P) involved in the acylation of Lipid A with palmitate. The increased hydrophobic interactions probably retard or abolish peptide insertion and pore formation (Guo et al. 1998).

S. aureus secretes staphylokinase that induces extracellular release of α-defensins from PMNs, subsequently neutralising their bacteriolytic properties by direct binding to the HDPs (Jin et al. 2004). Likewise, some highly virulent *Streptococcus pyogenes* strains produce an anionic extracellular protein, SIC (streptococcal inhibitor of complement), that inhibits LL-37, HNP1, and HBD1-3. *sic* is expressed at an early growth phase, suggesting that SIC will be secreted as soon as *S. pyogenes* starts growing on an epithelial surface. This may promote the early stages of infection by inactivating HDPs (Frick et al. 2003;Fernie-King et al. 2004). Increasing evidence indicates that proteolytic enzymes also play a role in HDP tolerance. In *S. aureus* the metalloproteinase aureolysin catalyses the simultaneous hydrolysis of several peptide bonds in LL-37 (Sieprawska-Lupa et al. 2004).

Intrinsic HDP resistance can also be caused by increased active transport of the peptide either into the cell for degradation or out of the cell. The SapABCDF (*sap*, sensitivity to antimicrobial peptides) complex in *S.* Typhimurium belongs to the ATP binding cassette (ABC) family of protein transporters. The predicted periplasmic location of the SapA component suggests that it may be responsible for the transportation of bound ligands *into* the cytoplasm of the cell (and away from their putative membrane targets) where they could be degraded by peptidases. Alternatively, by detecting the presence of toxic compounds, Sap could initiate a regulatory cascade resulting in the activation of the relevant peptide resistance determinants. Inactivation of *sap* resulted in increased sensitivity to protamine and the microbicidal compounds of human neutrophil granules (Parra-Lopez et al. 1993).

Shigella spp. is capable of down-regulating transcription of LL-37 and HBD-1 early during infection, which probably facilitates colonization of the intestinal mucosa (Islam et al. 2001).

2.5. Conclusions

The innate immune system combats invading pathogenic microorganisms through an array of effector molecules and one of the main contributors to these chemical defense lines are the host defense peptides, HDPs. HDPs can be divided into three classes based on their secondary structure. Across these three classes is a group of structurally heterogeneous peptides called cathelicidins. HDPs have direct antimicrobial actions and probably function as dirty drugs, acting on several microbial targets at the same time. We have shown that the HDPs used in this study have different mechanisms of action. In addition, HDPs have immunomodulatory actions that function to boost the immune system and thus contribute to infection-resolving immunity. However, pathogenic microorganisms have evolved various strategies to resist the antimicrobial activities of HDPs which contribute to their virulence.

3. Host defense peptides as novel antimicrobials

Host defense peptides have two possible applications in the therapy of infectious diseases. Their broad antimicrobial activity spectrum and rapid action make them attractive candidates as direct antimicrobial agents. In addition, their recently discovered immunomodulatory properties that stimulate the innate immune system to resolve infection make the HDPs an entirely new approach to treat infectious diseases. Together, this has prompted a massive interest in HDPs as a new generation of antimicrobials (Hancock and Sahl 2006). In addition to the therapeutic perspectives, the broader group of antimicrobial peptides (AMPs) are being researched as possible food preservatives (Potter et al. 2005).

3.1. Variation in tolerance of human pathogens to HDPs

The central dogma for HDPs is that they have been an effective part of the innate immune system for millions of years targeting all classes of microorganisms. However, a cardinal feature of pathogenic microorganisms is that they are able to circumvent the massive attack of these antimicrobial compounds and establish an infection. The importance of such intrinsic HDP resistance in virulence is verified by a number of studies of mutants with increased HDP susceptibility compared to their parental wild type, and subsequently attenuated virulence (Peschel et al. 2001;Abachin et al. 2002;Collins et al. 2002;Thedieck et al. 2006).

The antimicrobial activity of a given compound is indicated as either the minimum inhibitory concentration (MIC) determined in a broth microdilution assay, or the minimum effective concentration (MEC) determined in a radial diffusion assay. Several studies have demonstrated the broad spectrum of antimicrobial activity of HDPs against several pathogenic species. However, the majority of these studies have analyzed only one or a few strains within each species, and this strain is often a laboratory reference strain. Turner et al. showed that the human cathelicidin LL-37 and the defensin HNP-1 had considerable antimicrobial activity (MIC <10 µg/mL for LL-37 and MIC < 20 µg/mL for HNP-1) against *Staphylococcus aureus*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, vancomycin-resistant enterococci, and *Escherichia coli*. LL-37 was also active against the Gram-negative *Pseudomonas aeruginosa* and *Salmonella* Typhimurium (Turner et al. 1998). Human intestinal defensin 5 (HD-5) was shown to have broad spectrum activity (MIC < 10 µg/mL) against one strain of *L. monocytogenes*, *E. coli*, *S.* Typhimurium, and *C. albicans*, respectively (Porter et al. 1997). Protamine used in this study has also been demonstrated to inhibit both Gram-positive and Gram-negative food borne pathogens and food spoilage

bacteria (Truelstrup Hansen et al. 2001). The cathelicidin novispirin G10 has been shown to have potent antimicrobial activity against single strains of several Gram-positive and Gram-negative species (MECs ranging from of 0.4 – 11.4 µg/mL), including a *S. aureus*, *S. epidermidis*, *Enterococcus faecalis*, *E. coli, P. aeruginosa*, and *Proteus mirabilis* (Steinstraesser et al. 2002;Jacobsen et al. 2007).

A limited number of studies have investigated the natural variation in tolerance of several strains of the same species to HDPs. The fungal defensin plectasin used in this study showed potent antimicrobial activity against a number of Gram-positive pathogenic species. At least ten and up to 133 strains within each species were studied, including both methicillin sensitive and –resistant *S. aureus* (MICs < 32 µg/mL), penicillin sensitive and –resistant *Streptococcus pneumonia* (MICs < 8 µg/mL) and erythromycin sensitive and –resistant *S. pyogenes* (MICs < 0.5 µg/mL). The antibiotic resistant strains were not more tolerant to plectasin than the sensitive strains (Mygind et al. 2005). Likewise, Maisetta et al. found that the human β-defensin, HBD-3, was equally active against several strains of multidrug-resistant *S. aureus*, *E. faecium*, and *P. aeruginosa* (Maisetta et al. 2006). In contrast, Joly et al. tested the activity of HBD-2 and HBD-3 in a radial diffusion assay against a variety of oral pathogens, and included 3-4 strains for each species. They found considerable variation in tolerance to both peptides, which was strain rather than species specific. Within the same species, MIC ranged from 6.5 to > 250 µg/mL for HBD-2 and from 4.5 to > 250 µg/mL for HBD-3 (Joly et al. 2004).

The bacterial equivalent to the HDPs, the bacteriocins, are used as antimicrobial agents in the food industry and also have potential implication in the development of desirable flora in fermented foods (Cotter et al. 2005). Numerous reports exist on variations in tolerance of field isolates of *L. monocytogenes* to bacteriocins. 245 *L. monocytogenes* isolates of different origin have been shown to vary in tolerance to bavaricin-A (Larsen and Norrung 1993), 22 strains isolated from ewe's milk varied in tolerance to enterocin 4 (Rodriguiez et al. 1997), 381 *L. monocytogenes* strains of clinical or environmental origin showed variation in tolerance to pediocin PA-1, bavaricin A and nisin (Rasch and Knochel 1998), and 31 *Listeria* sp. of food origin, including 14 *L. monocytogenes* strains, varied in susceptibility to enterocin A, mesentericin Y105, divercin V41, and pediocin AcH. These four bacteriocins showed similar antimicrobial patterns, with some strains being fully resistant to all four compounds. This is of great concern with respect to their use as antimicrobial agents (Ennahar et al. 2000). The effect of bacteriocins on *S. aureus* strains have mainly focused on comparing antibiotic resistant and sensitive strains. Piper et al. found that groups of *S. aureus* strains with different antibiotic susceptibility patterns did not vary greatly in susceptibility to neither

nisin nor lacticin 3147. The MIC varied within a factor of two to four within each group (Piper et al. 2009), which is within the assay insecurity when doing two-fold dilution MIC determinations. Likewise, nisin caused extensive loss of viability in all strains of multidrug resistant staphylococci, *S. pneumoniae* and enterococci (Severina et al. 1998).

We speculated that any variation in virulence between strains of pathogenic bacteria could be explained by differences in tolerance to HDPs, since this might give the more tolerant strains the opportunity to establish a niche on the epithelial surface from where the infection can subsequently be initiated. The occurrence of such natural tolerant isolates would hamper the potential use of HDPs as antimicrobial therapeutics. Therefore, we examined the susceptibility to HDPs in a group of natural occurring isolates of *L. monocytogenes* and *S. aureus* that represent a broad spectrum of origins, sub types and phenotypic virulence-associated behavior. We used four model peptides, protamine, plectasin, novicidin and novispirin G10, which were chosen because they represent each of the three different classes of HDPs. In addition, as mentioned above, ATP leakage profiles suggest that they represent different mechanisms of action (Figure 2.4). We also included the human β-defensin HBD-3 against a subset of strains. HBD-3 is expressed at epithelial surfaces of both the skin and mucosa (Harder et al. 2001), and is thus highly relevant for both *L. monocytogenes* and *S. aureus*. We found no systematic differences in tolerance to the four model HDPs or HBD-3 between strains of *L. monocytogenes* and *S. aureus* (Table 3.1) (Gottlieb et al. 2008).

Correspondingly, another study has found that *L. monocytogenes* does not differ remarkably compared to the non-pathogenic species *L. innocua* and *L. ivanovii* with respect to tolerance to HDPs such as HBD-1, HBD-2, thionins, magainin, and protamine (Lopez-Solanilla et al. 2003). These results are physiologically logical, since HDPs are designed to target whatever pathogenic microorganism that enters the host organism.

Table 3.1: Origin, subtype and MIC values of the *Listeria monocytogenes* and *Staphylococcus aureus* strains used in the present study. Modified from (Gottlieb et al. 2008).

L. mono	Origin	Sero	Lin	MIC[a]						Ref
				Pro	Ple	NoC	NoS	HBD	H_2O_2	
La22	Cold smoked salmon	1/2a	2	16	128	4	64	16/16	0.23	(Vogel et al. 2001b)
V518a	Fish processing	4b	1	32	64	8	64	-	0.94	(Vogel et al. 2001b)
N53-1	Fish processing	1/2a	2	16	128	2	32	16/32	0.47	(Wulff et al. 2006)
No40-1	Fish processing	1/2a	2	16	128	4	64	-	0.47	(Wulff et al. 2006)
R479a	CS salmon	1/2a	2	16	128	4	64	-	0.47	(Vogel et al. 2001b)
O57	Gravad salmon	1/2a	2	16	128	4	128	-	0.47	(Embarek and Huss 1993)
H13-1	Fish processing	1/2a	2	16	128	4	64	-	0.94	(Wulff et al. 2006)
La111	Cold smoked salmon	1/2a	2	8	64	4	32	8/16	0.94	(Vogel et al. 2001a)
M103-1	Fish processing	1/2a	2	32	128	4	64	-	0.94	(Wulff et al. 2006)
EGD	Wildtype	1/2a	2	8	64	4	64	16/16	0.47	[b]
2315	EGD *sigB* del	1/2a	2	8	64	4	64	-	0.94	(Brondsted et al. 2003)
2317	EGD *prfA* del	1/2a	2	16	128	4	64	-	0.94	[b]
2375	EGD *perR* del	1/2a	2	32	64	4	32	-	0.94	(Rea et al. 2004)
2374	EGD *perR* ins	1/2a	2	32	128	4	32	-	0.94	(Rea et al. 2004)
2275	EGD *dps* del	1/2a	2	16	64	4	64	-	0.94	(Olsen et al. 2005a)
2307	EGD *resD* del	1/2a	2	8	128	2	32	-	0.12	[c]
LO28	Wildtype	1/2c	2	8	64	2	16	-	0.47	(Vazquez-Boland et al. 2001)
4666	Human clinical	1/2b	1	8	64	8	64	-	0.18	(Larsen et al. 2002)
4459	Human clinical	1/2a	2	16	128	4	32	-	0.23	(Larsen et al. 2002)
7418	Spread. sausage	1/2b	1	32	64	4	64	4/8	0.18	(Larsen et al. 2002)
4446	Human clinical	4b	1	16	64	4	64	16/16	0.47	(Larsen et al. 2002)
6895	Ham	1/2a	2	16	128	4	96	-	0.35	(Larsen et al. 2002)
7291	Pasta w chicken	4b	1	32	64	8	128	-	0.47	(Larsen et al. 2002)
4239	Human clinical	1/2a	2	32	64	4	64	-	0.23	(Larsen et al. 2002)
Scott A	Human clinical	4b	1	16	64	4	32	8/8	N.D.	[d]

S. aureus	Origin	*spa*	MIC						Ref
			Pro	Ple	NoC	NoS	HBD	H_2O_2	
8325-4	Wildtype	t211	16	32	8	128	32/32	0.47	(Novick 1967)
Sa113	Wildtype	t211	16	32	6	128	-	0.18	(Iordanescu and Surdeanu 1976)
Δ*mprF*	Sa113 *mprF* del	t211	8	4	1	8	-	0.12	(Peschel et al. 2001)
Δ*dltA*	Sa113 *dltA* ins	t211	8	2	0.5	2	-	0.23	(Peschel et al. 1999)
14943	Pork meat	t012	16	8	8	256	32/64	0.23	[e]
15033	Pork meat	t216	32	8	8	128	-	0.23	[e]
B31369	Human, clinical	t216	16	16	12	256	64/64	0.47	[e]
796	Pasta salad	t230	16	8	6	128	64/64	0.47	[e]
J15033	Human, clinical	t230	16	8	8	128	-	0.23	[e]
2148-jvi	Mastitis	t518	16	1	4	64	32/32	0.47	[e]
K3-B2	French cheese	t524	16	1	4	128	32/64	0.23	[e]
B29997	Human, clinical	t548	16	16	4	128	-	0.23	[e]
KES 439	Human, clinical	Uk[f]	32	2	4	128	-	0.23	(Gjødsbøl et al. 2006)
KES 626	Human, clinical	t1269	16	1	4	64	32/64	0.18	(Gjødsbøl et al. 2006)
KES 735	Human, clinical	Uk[f]	16	16	4	128	-	0.47	(Gjødsbøl et al. 2006)
KES 855	Human, clinical	t339	16	16	4	64	-	0.23	(Gjødsbøl et al. 2006)

[a] MIC values are given in µg/ml for the five human defense peptides and in % (V/V) for H_2O_2.

[b] The strains were kindly provided by Werner Goebel, University of Würzburg

[c] The strain was kindly provided by Marianne Halberg Larsen, University of Copenhagen, Faculty of Life Sciences.

[d] The strain was kindly provided by Campden Food and Drink Association, UK.

[e] The strains were kindly provided by Jørgen Leisner, University of Copenhagen, Faculty of Life Sciences.

[f] Uk: Unknown.

In contrast, others have found that the susceptibility of clinical isolates of *S. aureus* (both MRSA and MSSA) to the two human HDPs, HBD-3 and hCAP18 varied among the strains both when assayed as percent survival of a 2 hour HDP treatment or when assayed regarding their susceptibility to different combinations of peptides (Midorikawa et al. 2003). Also, differences between two *S. aureus* reference strains have been observed in the susceptibility to HBD-2 and the murine cathelin-related antimicrobial peptide, mCRAMP (Clarke et al. 2007). The discrepancy to our results can be related to the fact that they examined the percent survival of initial inoculums after 2 hours of treatment, whereas we examined the inhibitory effect on growth after 24 hours. It has previously been shown that there are differences between *L. monocytogenes* EGD and sigB mutant in bacteriocin tolerance when assayed in "real time" as opposed to endpoint assays such as MIC determinations (Begley et al. 2006). However, we have not observed differences between *L. monocytogenes* EGD and its *sigB* mutant or between wildtype strains when compared in realtime (Figures 3.1 and 3.2) (Gottlieb and Gram 2009).

3.2. Stress response and HDP susceptibility

To evaluate the potential role of HDPs in antimicrobial therapy, knowledge about how microorganisms react to treatment with HDPs is necessary. During the course of infection, pathogenic bacteria are exposed to an array of stresses, including the HDPs of the innate immune defense. Environmental stresses as met both before infection, e.g. in foods, as well as during the infectious process might elicit a stress response in the pathogenic bacteria that could prime the pathogen for the subsequent stages of infection.

3.2.1. Potential role for SigmaB in growth and survival in the presence of HDPs

A common trait for all bacteria is the use of alternative sigma factors to react to and cope with stressful conditions. Indeed, *S. aureus* grown in the presence of carbonate, mimicking the mammalian ionic environment, had a ten-fold decreased expression of *sigB* encoding the Gram-positive alternative sigma factor SigmaB. This correlated with an increased susceptibility to a number of HDPs, including LL-37 and HBD-2 (Dorschner et al. 2006) suggesting that *sigB* is involved in tolerance to HDPs. Likewise, the Gram-negative alternative sigma factor, SigmaE, is involved in the resistance of the two human enteric pathogens *Salmonella enterica* serovar Typhimurium and *Vibrio cholerae* to P2, a derivate of a human HDP (Humphreys et al. 1999;Crouch et al. 2005;Mathur et al. 2007). Also, SigmaB has been shown to be involved in the response of *L. monocytogenes* to both the bacteriocins nisin and lacticin 3147, and the conventional antibiotics penicillin and ampicillin (Begley et al.

2006;Palmer et al. 2009). Surprisingly, Begley et al. found that a *sigB* mutation did not affect the bacteriocin tolerance in endpoint assays, while survival assays showed that a *sigB* mutation decreased the tolerance to nisin (Begley et al. 2006). In contrast, Palmer et al. found that a *sigB* mutation increased the survival of nisin treatment (Palmer et al. 2009).

We hypothesized that SigmaB was involved in the tolerance to HDPs in *L. monocytogenes* and investigated the growth and survival of *L. monocytogenes* EGD and *ΔsigB* in the presence of HDPs. Like Begley et al. (2006) we did not observe differences in tolerance to HDPs between *L. monocytogenes* EGD and *ΔsigB* in endpoint assays (Table 3.1) (Gottlieb et al. 2008). In contrast, we found that there was no difference between the wildtype and the *sigB* mutant with respect to both growth at sublethal concentrations of plectasin or novicidin or the survival of lethal concentrations of plectasin (Figure 3.1) (Gottlieb and Gram 2009). The discrepancy could be due to different mechanisms of action of the antimicrobials investigated, as one could speculate that this would confer different kind of stresses and hence different responses.

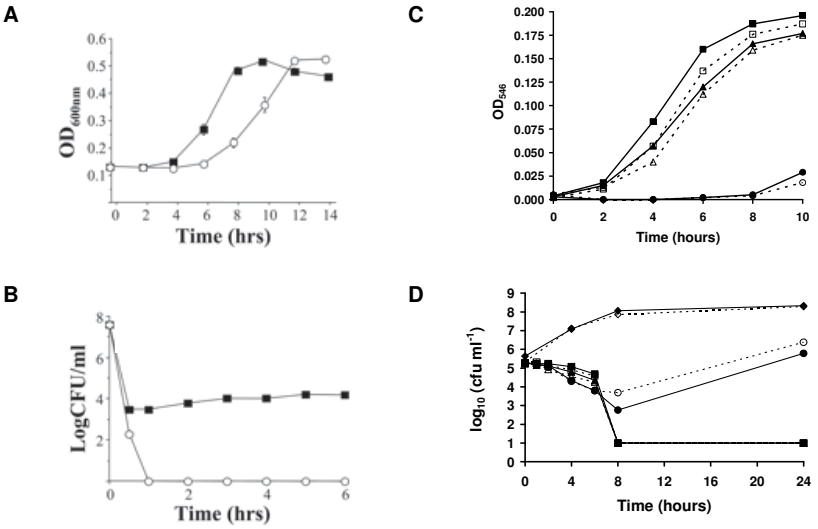


Figure 3.1: (A-B) Growth and survival of *L. monocytogenes* 10403S (■) and *ΔsigB* (○) in the presence of nisin. Adapted from (Begley et al. 2006). (C) Growth of *L. monocytogenes* EGD (closed symbols) and *ΔsigB* (open symbols) in the presence of plectasin (▲△), novicidin (●○), or buffer (■□). (D) Survival of *L. monocytogenes* EGD (closed symbols) and *ΔsigB* (open symbols) in the presence of 256 µg/ml (■□), 128 µg/ml (▲△), or 64 µg/ml (●○) of plectasin or peptide dilution buffer (♦◊). Adapted from (Gottlieb and Gram 2009).

To analyze whether *sigB* is in fact induced during HDP exposure, an agar-based assay could be applied (Kastbjerg et al. 2009). The principle of the assay is that gene expression is monitored through β-galactosidase production from strains carrying a *sigB* promoter:*lacZ*-fusion that is cast into an agar plate. HDPs could then be added to wells in the agar and the color of the colonies close to the inhibition zone reveals whether *sigB* is induced or repressed. Also, Kastbjerg et al. (2009) demonstrated that virulence gene promoter expression was up- or down-regulated differently in response to treatment with various disinfectants depending on the active compound, hence it could be speculated that different classes of HDPs would induce expression of *sigB* differently depending on the mechanism of action.

3.2.2. Effect of exposure to environmental stress conditions on tolerance to HDPs

L. monocytogenes is capable of survival and growth under adverse conditions normally used in food conservation, including low pH, high salt concentration and low temperatures (Farber and Peterkin 1991). SigmaB is a major contributor to bacterial survival under adverse growth conditions such as high osmolarity, acid stress, cold stress, ethanol, carbon starvation, and oxidizing conditions (Wiedmann et al. 1998;Becker et al. 1998;Ferreira et al. 2001;Sue et al. 2004;Chaturongakul and Boor 2006). SigmaB also regulates the expression of several virulence genes, including bile salt hydrolase, *bsh* (protecting against the toxic effect of bile) and the invasion-mediating internalins, *inlA* and *inlB* (Kazmierczak et al. 2003;Sue et al. 2004). Furthermore, SigmaB is involved in the invasion of *L. monocytogenes* into Caco-2 cells (Kim et al. 2005;Garner et al. 2006a). Interestingly, the relative importance of SigmaB in the stress response differ between strains of *L. monocytogenes*, and may be related to the serotype (Moorhead and Dykes 2003;Severino et al. 2007), suggesting that SigmaB could contribute to strain differences in stress tolerance and virulence.

Exposure to sub-lethal levels of such environmental stress conditions have been shown to induce a stress response in *L. monocytogenes* that renders it more tolerant to lethal levels and to other forms of stress, a phenomenon known as stress-hardening (O'Driscoll et al. 1996;Lou and Yousef 1997;Begley et al. 2002). Especially, this cross-protection might result in pathogens adapted to e.g. food-related stress conditions with increased resistance to the stresses met during infection. Indeed, adaptation to acidic, NaCl, or oxygen restriction stress prior to infection of Caco-2 cells increased the invasive ability of *L. monocytogenes* (Garner et al. 2006b;Andersen et al. 2007;Werbrouck et al. 2009;Olesen et al. 2009). Acid adaptation also increased the survival in macrophages (Conte et al. 2002), suggesting that virulence-associated characteristics are likely to be affected by food-related stress conditions. Thus,

besides the enhanced survival in foods which increases the chance (or rather, the risk) of ingesting an infectious dose of *L. monocytogenes*, such stress adaptation also augments the virulence of the target organism (Hill et al. 2002;Garner et al. 2006b). On the other hand, the stress conditions found in a potted minced pork product, rillettes, that has been implicated in cases of listeriosis, actually limited the *in vitro* virulence phenotype of *L. monocytogenes* (Midelet-Bourdin et al. 2006).

In addition to the *in vitro* virulence phenotype, adaptation to 6.5% NaCl or 5°C for 1 hour have been shown to increase the tolerance to the bacteriocin pediocin PA-1 (Jydegaard et al. 2000). Likewise, acid adaptation of *L. monocytogenes* at pH 5.5 (adjusted with lactic acid) for 1 hour significantly increased the resistance to nisin, whereas the protective effect of acid adaptation was much less pronounced for treatment with lacticin 3147 (van Schaik et al. 1999). This indicates that stress-hardening of *L. monocytogenes* strains in a food matrix may hamper the effect of bacteriocins as a food preservation factor (van Schaik et al. 1999).

We hypothesized that exposure to food-related environmental stress factors could increase the tolerance of *L. monocytogenes* to subsequent HDP exposure. We have investigated the effect of three food-related stress factors, 5% NaCl, pH 5.5, and growth at low temperature (5°C/10°C) on tolerance to subsequent HDP treatment. We found that in the experimental design of this study, these food-related factors did not affect HDP tolerance, neither when treated with single peptides or in combination of several. Also, there was no difference between the three strains used, representing different origins and serotypes. Representative results from exposure to pH 5.5 is shown in Figure 3.2 (Gottlieb and Gram 2009).

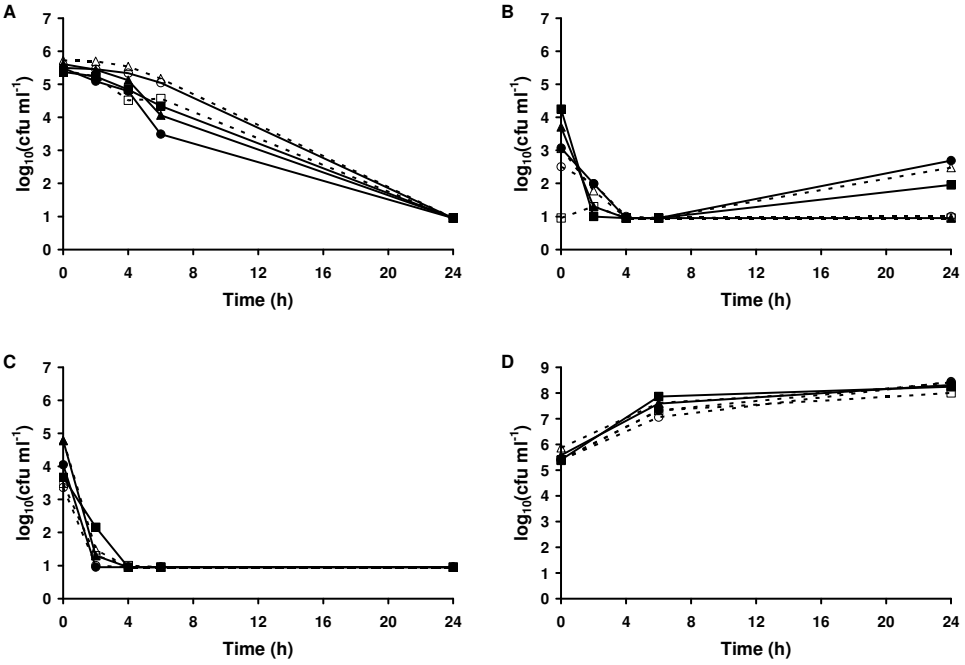


Figure 3.2: Treatment of *L. monocytogenes* strains with plectasin, novicidin or a combination after pretreatment with pH 5.5 or pH 7.0. Strains were grown in TSB 1% glucose pH 5.5 (closed symbols, straight lines) or pH 7.0 (open symbols, dotted lines) in two successive inoculations, harvested, washed, and resuspended in MHB pH 7.4 to app. 5×10^5 CFU/mL prior to treatment with (a) plectasin, (b) novicidin, (c) a combination of plectasin and novicidin, or (d) peptide dilution buffer. Strains and stress conditions: (■) EGD pH 5.5, (□) EGD pH 7.0, (▲) 4446 pH 5.5, (△) 4446 pH 7.0, (●) N53-1 pH 5.5, and (○) N53-1 pH 7.0. From (Gottlieb and Gram 2009).

The discrepancies between our results and those regarding stress adaptation and bacteriocin tolerance (van Schaik et al. 1999;Jydegaard et al. 2000) could be due to different mechanisms of action between the bacteriocins and the HDPs used in this study. However, it could also be explained by the use of short term exposure as opposed to long term exposure to the stress factors (Olesen et al. 2009), the use of organic versus inorganic acid to lower the pH (Phan-Thanh et al. 2000), or the growth phase of the bacteria (O'Driscoll et al. 1996).

Together, our results suggest that the potential use of HDPs will not be hampered by a stress response in pathogenic bacteria that would render them more tolerant to HDPs and thus lowering the therapeutic effect of HDPs. However, it is possible that the stresses met during gastric passage might prime *L. monocytogenes* for the next phase of infection, which

includes the HDPs at the intestinal mucosa and the subsequent systemic invasive disease (Sleator et al. 2009).

3.3. Potential development of resistance

The development of resistance towards HDPs is considered highly unlikely for two main reasons. First, the HDPs targets fundamental features of the bacteria that will be hard to change without a large fitness cost to the bacterium. Second, the great diversity of HDPs together with the simultaneous release of several different kinds of HDPs at the site of infection and with different mechanisms of action impedes the probability of evolution of resistance in the bacterial population (Zasloff 2002). However, if or when the HDPs are taken out of their natural environment and used as antimicrobials in a clinical setting, they will confer a specific and continued selection pressure on the bacterial population that eventually might lead to the evolution of resistance. This could have more severe implications if it will lead to cross-resistance to human innate antimicrobial peptides (Bell and Gouyon 2003;Perron et al. 2006). A better understanding of the molecular basis of bacterial resistance to HDPs may provide clues on how to design synthetic peptides to circumvent potential resistance problems (Zaiou 2007).

3.3.1. Bacterial sensory systems involved in HDP resistance

A cardinal feature of pathogenic microorganisms is the occurrence of intrinsic resistance mechanisms to HDPs that are essential for pathogenesis as described in section 2.4. Since the constitutive production of such intrinsic resistance mechanisms probably represents a significant energy burden to the bacteria, the expression is restricted to times when HDPs are present in the environment. Consequently, pathogenic bacteria have evolved regulatory mechanisms that sense the presence of HDPs (or other molecular markers of intra-host existence) and control the expression of resistance genes accordingly. Two-component systems (TCS) are basic stimulus-response systems that allow bacteria to sense and respond to changes in the environment (Stock et al. 2000). Several of such two-component systems have been described to be involved in bacterial sensing and responding to antimicrobial peptides as well as virulence. The VirRS TCS in *L. monocytogenes* controls the expression of the *dlt* operon and *mprF*, strongly suggesting an important role in regulation of *L. monocytogenes* resistance to HDPs (Mandin et al. 2005). Likewise, the CesRK system is involved in virulence and resistance to β-lactam antibiotics (Kallipolitis and Ingmer 2001;Kallipolitis et al. 2003). Inactivation of the staphylococcal ApsRS system resulted in increased susceptibility to HDPs such as HBD-3 and LL-37 (Li et al. 2007). Likewise, the

staphylococcal GraRS also contributes to resistance to LL-37 as well as virulence *in vitro* in neutrophils and *in vivo* in mice (Kraus et al. 2008). PhoPQ controls inducible resistance to HDPs in a number of Gram-negative species (Bader et al. 2005). Hence, such bacterial sensory systems could be potential targets for novel antimicrobial therapeutics (Brodsky and Gunn 2005).

Surprisingly, inactivation of the heme sensor TCS, HssRS, in *S. aureus* resulted in enhanced virulence. This correlated with an increased secretion of virulence factors with known immunomodulatory functions and hence a reduced innate immune response in the host (Torres et al. 2007). Also, just as the carbonate-mediated down-regulation of *sigB* in *S. aureus* as mentioned earlier (Dorschner et al. 2006), it could be speculated that also other host factors could induce down-regulation of virulence-associated genes. Hence, the inactivation of genes involved in sensing the mammalian environment could lead to a HDP-resistant phenotype. We investigated if resistance to the defensin plectasin could be induced by transposon mutagenesis in *S. aureus* and *L. monocytogenes*. While we did not find any mutants in *L. monocytogenes*, two *S. aureus* mutants with the transposon inserted in the heme response regulator *hssR* were identified. The mutation caused increased tolerance to plectasin and the plectasin-like defensin eurocin, but not to other classes of HDPs or conventional antibiotics (Thomsen et al. 2009).

A mechanistic explanation for the intuitively illogical phenotype of *hssRS* mutants is given in Figure 3.3. Since heme is a central component of hemoglobin and myoglobin, it can function as a molecular marker that can potentially be exploited by infecting bacteria to sense that the surface tissues of the host have been breached, allowing them to adjust to an internal life cycle. The inability to sense and excrete surplus heme could result in a stress response in *S. aureus* leading to increased virulence factor secretion (Torres et al. 2007). Among these could be proteins that neutralize plectasin and plectasin-like compounds.

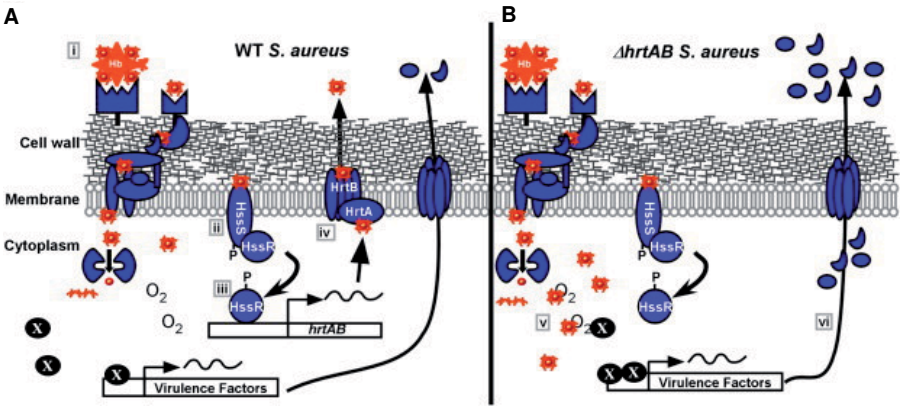


Figure 3.3: Model for the role of HssRS and HssAB in *S. aureus* pathogenesis. (A) In wildtype *S. aureus* HssS sense internalized heme, activates HssR that bind the *htrAB* promoter activating the expression of *htrAB*. HtrAB then mediates efflux of surplus heme that is otherwise toxic to the cell. (B) Inactivation of *hrtAB*, or in our case *hssR*, leads to accumulation of surplus heme in the cytoplasm. This activates a stress response in *S. aureus* that increases the expression and/or secretion of virulence factors. These virulence factors could include proteins that neutralize plectasin. From (Torres et al. 2007).

Interestingly, *L. monocytogenes* LO28 RR23, carrying an insertional deletion in the putative *L. monocytogenes hssR* homologue *rr23*, did not have increased tolerance to plectasin. Significant interspecies differences in the HDP-TCS interaction have been observed (Li et al. 2007), which could explain the different phenotypes of *S. aureus* and *L. monocytogenes* LO28 observed in this study.

Also, as is becoming increasingly evident, the environmental role of conventional antibiotics is not only as antimicrobials. They also serve signaling purposes in the low concentrations found naturally, allowing microorganisms in an ecosystem to communicate with each other (Goh et al. 2002). Consequently, in the complex world of microbial ecosystems, the inhabitants probably depend on compounds produced by other members of the community, to turn on specific metabolic pathways, and start biofilm formation (López et al. 2009) to orchestrate the growth of the community (Goh et al. 2002). The presence of intrinsic resistance genes has thus been selected in their hosts as a way of sensing their surroundings (Martinez 2009). As *L. monocytogenes* is ubiquitously found in the environment living as a saprophyte on decaying matter, and plectasin is isolated from the saprophytic ascomycete *Pseudoplectania nigrella*, it could be speculated that *L. monocytogenes* unlike *S. aureus* have already encountered plectasin-like compounds in its ecosystem. Hence, *L. monocytogenes* could harbor intrinsic tolerance mechanisms to plectasin and similar

compounds and have therefore already evolved a maximum ability for sensing plectasin, explaining why we can only induce tolerance in *S. aureus*. This is consistent with the finding that *L. monocytogenes* is generally more tolerant to plectasin than *S. aureus* (Mygind et al. 2005;Gottlieb et al. 2008)

3.3.2. Evolution of resistance to HDPs through cumulative changes

As a consequence of the ongoing interplay between microbial infection strategies on the one hand and host defense strategies on the other, the host-pathogen co-evolution has resulted in a balance between the host repertoire of HDPs and the microbial repertoire of resistance mechanisms (Peschel and Sahl 2006). HDP genes are among the most rapidly evolving groups of mammalian proteins, which suggests a highly dynamic system in which virulence and resistance continually shift as the result of co-evolutionary arms race between the host and their bacterial pathogens (Vanhoye et al. 2003;Peschel and Sahl 2006;Hancock and Sahl 2006).

As opposed to conventional antibiotics that often have a single, high-affinity target and a single mode of action, the evolutionary concept of antimicrobial peptides appears to be as “dirty drugs” targeting many biological functions but with modest potency, thus minimizing the risk of development of resistance (Peschel and Sahl 2006). In addition, the simultaneous release of several HDPs with different mechanisms of action at the site of infection also impedes the development of resistance (Hornef et al. 2002;Peschel and Sahl 2006). Also, the continual presence of HDPs in all host environments has not led to generalized or high level of resistance (Hancock 2003).

The presence of multiple HDP targets in bacteria makes development of resistance difficult as it would require several mutations in target molecules. Hence, evolution of resistance most probably will have to occur through cumulative changes involving several loci, which will require a long selection period of serial transfers of cultures to medium with gradually increasing HDP concentration to allow mutants with mildly beneficial effects to appear and spread in the population (Perron et al. 2006).

Repeated subculture of *S. aureus* and *P. aeruginosa* in broth containing ½ MIC of protegrin-1 for 18 serial passages failed to induce resistance in either organism (Steinberg et al. 1997). Likewise, up to 14 serial passages of several bacterial species, including *S. aureus* and *P. aeruginosa*, on plates containing ½ MIC pexiganan did not increase the tolerance, indicating that *in vitro* resistance acquired by the selection of mutations within the population of a given bacterial isolate does not occur with pexiganan (Ge et al. 1999). In contrast, both studies

demonstrated that the same short-term selection experiments induced resistance to conventional antibiotics (Steinberg et al. 1997;Ge et al. 1999). However, on a long-term basis high levels of heritable resistance to pexiganan evolved in populations of both *Escherichia coli* and *Pseudomonas fluorescens* when passaged for 100 daily transfers (corresponding to 6-700 generations) in increasing concentrations (Perron et al. 2006). The reason why this has not been seen in nature can be that under natural conditions, a population is exposed to an array of stresses that change from generation to generation. In the laboratory, and when antimicrobial agents are used in the clinical setting, the population is exposed to a particular stress that limits its growth, generation after generation, creating an intense selection that in turn is very likely to cause resistance in the population (Perron et al. 2006).

3.4. Conclusions

The data produced in this study does not speak against the use of HDPs as alternative antimicrobial therapeutics. We have not seen natural tolerant isolates in a broad collection of *L. monocytogenes* and *S. aureus* strains to four model HDPs representing each of the three classes. Furthermore, exposure of *L. monocytogenes* to food-related environmental stress conditions did not alter the response to subsequent HDP treatment. Finally, using transposon mutagenesis we identified a plectasin-tolerant *S. aureus* mutant with a two- to four-fold increased tolerance but we not find tolerant *L. monocytogenes* mutants. However, selection experiments allowing mildly beneficial mutations to accumulate have been shown to provoke HDP resistant mutants.

4. *Staphylococcus aureus* and *Listeria monocytogenes* – Gram-positive pathogenic bacteria

S. aureus and *L. monocytogenes* are closely related bacterial species belonging to the class of Bacilli, which comprises a group of Gram-positive bacteria with low G+C also including *Bacillus* and lactic acid bacteria. *S. aureus* and *L. monocytogenes* are pathogenic bacteria that represent different ecological niches, routes of infection and disease spectra.

4.1. Staphylococcus aureus

4.1.1. General characteristics and natural niches

The genus *Staphylococcus* includes more than 30 different species, of which two, *S. aureus* and *S. epidermidis*, are important with regard to human disease. *Staphylococcus* species can be subdivided into two groups on the basis of the ability to produce the enzyme coagulase. *S. aureus* is the principle species of the coagulase-positive and *S. epidermidis* of the coagulase-negative group (Lowy 1998).

S. aureus is a Gram-positive, non-sporeforming, nonmotile, facultative anaerobic, clusterforming coccus. The natural reservoir of *S. aureus* is the skin and mucus membranes of vertebrates (Lowy 1998;Ben Zakour et al. 2008). *S. aureus* shows some degree of host species specificity, and five biotypes have been proposed to divide *S. aureus* strains according to host origin (Hájek and Marsálek 1971;Devriese and Oeding 1976). In addition, *S. aureus* has the ability to asymptomatically colonize healthy individuals, the ecological niche being the anterior nares. Three patterns of carriage exist. Approximately 20% of the population is persistent carriers that almost always carry one type of strain, the majority of the population (60%) is intermittent carriers that harbor different *S. aureus* strains from time to time, and finally 20% of the population almost never carry *S. aureus* (Kluytmans et al. 1997).

S. aureus can grow between 7 and 48 °C, in the presence of up to 10% NaCl and at pH values ranging from 4.0 to 9.8 (Jay 1996). *S. aureus* is resistant to drying and desiccation (Rountree 1963;Tolba et al. 2007) meaning that it can survive on various surfaces outside its host habitat.

To distinguish strains of *S. aureus* and categorize them into groups that correlate with epidemiological origin, various molecular typing methods have been used. DNA sequence-based approaches have the advantage of being easy to perform and share between

laboratories. The *S. aureus* strains in this study have been typed using the *spa*-typing technique that is based on DNA sequencing of repeat regions of the *spa* gene (protein A) (Shopsin et al. 1999). *spa* typing has been shown to perform equally well as multi-locus enzyme electrophoresis (MLEE) and pulsed field gel electrophoresis (PFGE) with respect to discriminatory power and phylogeny (Koreen et al. 2004).

4.1.2. Routes of contamination and infection

S. aureus was described for the first time in 1880 by Ogston as the causative agent of sepsis and abscesses (Ogston 1880) and was identified to also be an animal pathogen in sheep (Nocard 1887) and cattle (Guillebeau 1890). The natural reservoir being vertebrates, *S. aureus* is either a hospital- or community-acquired pathogen (Lowy 1998). It is normally transferred from infected persons, either with overt clinical disease or asymptomatic carriers, to new hosts within the same species. However, cross-infections between host species have been observed, especially between domesticated animals and humans (Rodgers et al. 1999;Simoons-Smit et al. 2000;van Leeuwen et al. 2005). The ability to survive on various surfaces such as hospital bed linen and coins for prolonged periods increases the chance of transmission to a new host (Rountree 1963;Tolba et al. 2007). While persistent carriage seems to have a protective effect for acquisition of other strains during hospitalization (Kluytmans et al. 1997), it also poses a risk. It was found that carriers had a three-fold increased risk compared to non-carriers of acquiring nosocomial *S. aureus* bacteraemia, with endogenous strains being responsible for the bacteremia in 80% of the cases (Wertheim et al. 2004). Genetic analysis of both carriage and invasive disease isolates showed that isolates of both groups were represented in the same genetic clusters, suggesting that essentially any *S. aureus* genotype carried by humans can cause invasive disease (Melles et al. 2004). Thus, *S. aureus* is an opportunistic pathogen. Interestingly, elimination of nasal carriage by topical administration of antibiotic also eliminated *S. aureus* on the hands, thereby reducing the risk of contamination (Reagan et al. 1991).

S. aureus is the cause of an array of disease mechanisms in humans, ranging from relatively mild, superficial skin infections manifesting as boils to more serious infections such as pneumonia, meningitis, endocarditis, osteomyelitis, and systemic septicemia. In addition, the production of exotoxins can cause food poisoning, toxic shock syndrome and scalded skin syndrome (Lowy 1998).

Staphylococcal infection is initiated when *S. aureus* is introduced to the underlying tissues whenever the skin or mucosal barrier is damaged. The portal of entry can also be a hair follicle, a needle stick, or a surgical wound. Other sites of entry include the respiratory tract,

probably when immune defenses are impaired due to other infections such as influenza (Lowy 1998). Also, the production of enterotoxins in foods due to *S. aureus* contamination and growth causes the gastrointestinal tract to be not as much an entry site for *S. aureus* cells but for the enterotoxins, leading to diarrhea and vomiting (Marrack and Kappler 1990).

Immediately after *S. aureus* entry, neutrophils are recruited to the site. The establishment of an inflammation serves to contain the infection locally and leads to the formation of pus-filled abscesses. If the inflammation fails to contain the infection, *S. aureus* gains access to the bloodstream, and the resulting bacteremia may cause seeding of *S. aureus* in other tissues including heart (causing endocarditis), the bones (causing osteomyelitis), or the joints (causing septic arthritis) (Lowy 1998). The sequence of events of staphylococcal invasion of tissues is illustrated in Figure 4.1.

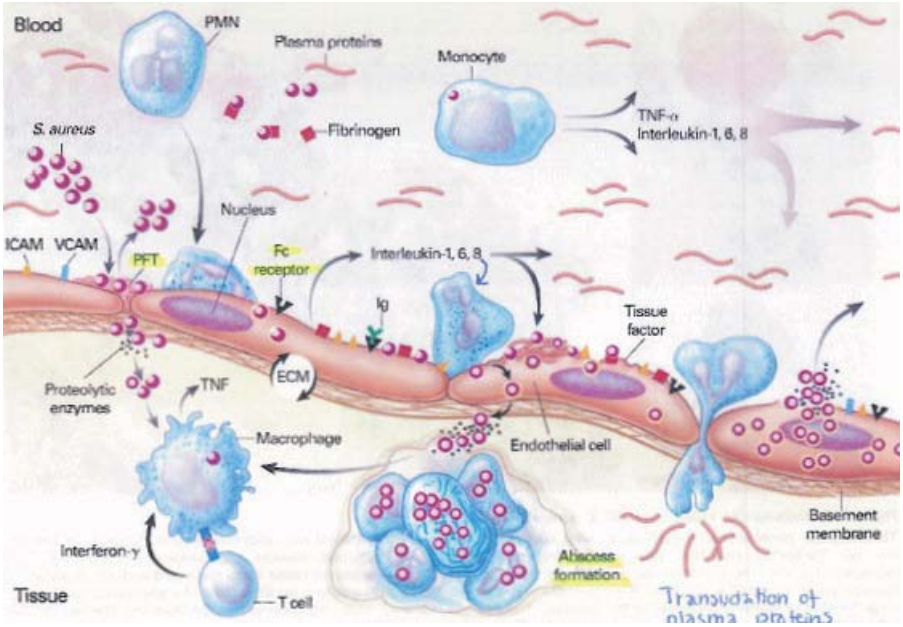


Figure 4.1: Course of infection of staphylococcal invasion of tissues. *S. aureus* circulating in the blood binds to sites of vascular damage and is phagocytosed by endothelial cells. The release of proteolytic enzymes facilitates the spread to adjoining tissues where abscess formation occurs. Infection of endothelial cells also induces expression of adhesion molecules and release of cytokines, resulting in recruitment of leukocytes to the site of infection and contributes to the manifestation of sepsis. From (Lowy 1998).

The tissue tropism of *S. aureus* may be mediated by staphylococcal surface proteins that can bind extracellular matrix molecules such as laminin, fibronectin, or collagen that are exposed due to vascular damage (Patti et al. 1994).

Although *S. aureus* is traditionally considered as an extracellular pathogen as opposed to *L. monocytogenes*, endothelial cells have been demonstrated to actively phagocytose *S. aureus* (Ogawa et al. 1985;Hamill et al. 1986). Also epithelial cells (Almeida et al. 1996), osteoclasts and fibroblasts are able to internalize *S. aureus* (Hudson et al. 1995).

In addition to the tissue damage induced by *S. aureus* itself, the bacterium also secretes an array of exotoxins. The membrane-damaging cytotoxins, α-toxin (α-hemolysin), β-toxin, δ-toxin, and the Panton-Valentine leukocidin, cause lysis of various host cells. The staphylococcal enterotoxins A, B, C, and D (SE-A – SE-D) cause vomiting and diarrhea when ingested in *S. aureus* contaminated food and can also cause toxic shock syndrome (TSS) when expressed by systemic *S. aureus* (Humphreys et al. 1989;Marrack and Kappler 1990). Both SE and toxic shock syndrome toxin, TSST-1, act as superantigens causing massive, unspecific stimulation of T-cells. This results in the release of large amounts of cytokines, accounting for the symptoms of TSS. Finally, two exfoliatin toxins, ETA and ETB, cause scalded skin syndrome (Lowy 1998).

4.1.3. Virulence factors in *S. aureus*

As seen in Figure 4.1, the pathogenesis for the majority of the diseases caused by *S. aureus* is multifactorial and hence is caused by the coordinated action of an array of virulence factors, with the only exception being diseases caused by specific toxins (Fournier and Philpott 2005).

The virulence factors of *S. aureus* can be broadly categorized by their function during the infectious process (see also Figure 4.2):

- Factors that promote colonization of host tissues
- Factors that promote bacterial invasion of host tissues
- Factors that contribute to evasion or survival of phagocytosis
    - Prevent immunological recognition
    - Prevent phagocytosis (including cytolytic toxins)
    - Enhance survival inside phagocytes
- Factors that otherwise damage host tissues or provoke symptoms of disease (toxins)

Colonization of host tissues is mediated by several staphylococcal surface proteins expressed during the exponential growth phase that are believed to be involved in the adhesion to the host tissue. These surface proteins, such as elastin-binding protein, collagen-binding protein and fibronectin-binding protein, bind extracellular matrix molecules via their N-terminal ligand-binding domain and have been designated microbial-surface components recognising adhesive matrix molecules (MSCRAMM) (Lowy 1998;Foster 2005).

To facilitate invasion, *S. aureus* also produces a number of excreted proteins, especially during stationary growth phase, which degrade host tissues and cells enabling the bacteria to invade and spread. These include the cytolytic toxins and proteases, lipases, and hyalorunidase (Foster 2005).

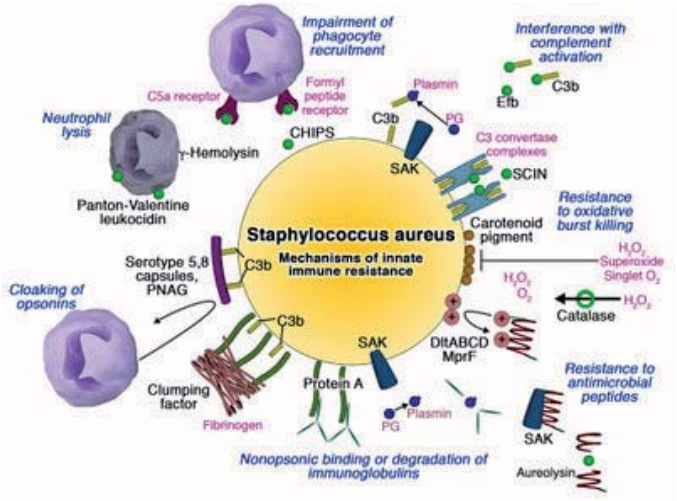


Figure 4.2: Virulence factors of *S. aureus* that contribute to host tissue invasion and immune evasion. From http://schaechter.asmblog.org/.a/6a00d8341c5e1453ef01127918d7f028a4-400wi (2/11-2009).

Once inside the host tissue, *S. aureus* is confronted by neutrophils and macrophages that are recruited to the site of entry by a gradient of chemoattractants that have been secreted by the damaged tissue. *S. aureus* inhibits chemotaxis by secreting proteins such as chemotaxis inhibitory protein of staphylococci (CHIPS) and the extracellular adhesion protein (eap) which block the host receptors involved in chemotaxis (Foster 2005;Rooijakkers et al. 2005b). Also, the production of the cytotoxins, α-toxin, γ-hemolysin, and Panton-Valentine leukocidin, enables *S. aureus* to lyse and kill phagocytic cells before they can interact with the bacteria (Bhakdi et al. 1989;Foster 2005).

Protein A has anti-phagocytic properties due to binding of the Fc region of immunoglobulin G, which is also recognised by the neutrophils (Lowy 1998;Foster 2005). Clumping factor (ClfA) binds and causes coating of the bacterial surface with fibrinogen, thereby inhibiting the deposition of or access to opsonins on the bacterial surface (McDevitt et al. 1997;Palmqvist et al. 2004). Likewise, complement factors on the bacterial surface are inaccessible to the neutrophil complement receptors due to capsular polysaccharides (Thakker et al. 1998). In addition, cell-bound staphylokinase causes cleavage of complement and IgG (Rooijakkers et al. 2005a).

Inside phagocytes, *S. aureus* withstands the oxidative burst through the action of catalase and carotenoid (Liu et al. 2005). In addition, the secretion of staphylokinase that binds defensins (Jin et al. 2004) and aureolysin that cleaves LL-37 (Sieprawska-Lupa et al. 2004) as described in chapter 2 contribute to staphylococcal survival of the innate antimicrobial attack.

4.1.4. Assessment of virulence potential

Virulence is a multifactorial phenomenon involving numerous factors from both the host and the invading pathogen. Factors such as age, gender, genetics, and the general immune status affect the host susceptibility. The pathogen usually produces an array of virulence factors, i.e. components that determine its capacity to cause disease but are not required for its viability. The true virulence of an organism (a strain) can only be determined in a natural situation of host infection, and may vary depending on the type of infection studied. However, single steps of the infection can be studied and be indicative of virulence potential.

Since neutrophils and macrophages are the first cells to arrive at the site of *S. aureus* infection (Foster 2005;Rooijakkers et al. 2005b), the virulence potential of *S. aureus* very much depends on the ability of *S. aureus* to evade the neutrophil attack. Indeed, enhanced strain virulence determined *in vivo* in mouse infection assays is linked to – or results from – the ability to evade killing by human neutrophils *in vitro* (Voyich et al. 2005). Therefore, we tested the collection of *S. aureus* strains for the phenotypic expression of several of the virulence factors described above, and also tested for the production of enterotoxins and TSST-1 (Figure 4.3 and Table 4.1).

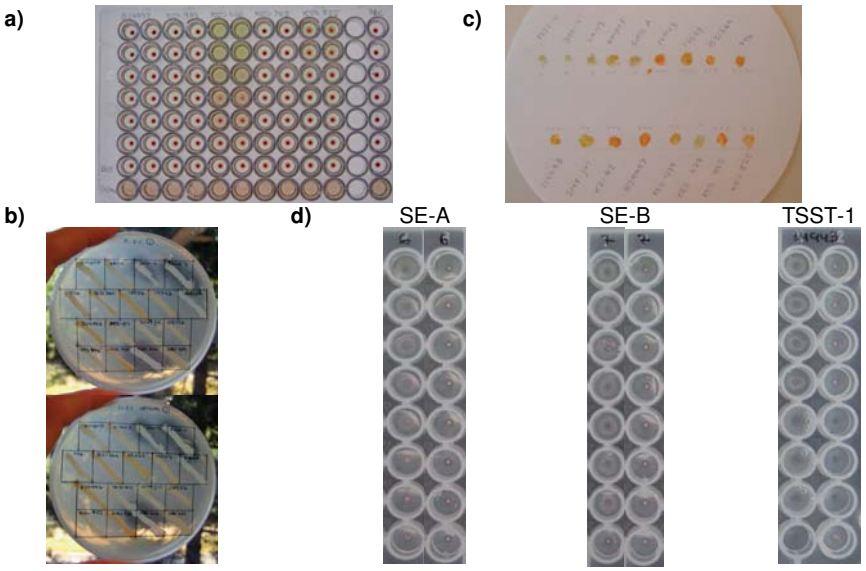


Figure 4.3: Determination of extracellular virulence factors in *S. aureus*. (a) Hemolysis. (b) Carotenoid production. (c) Staphylokinase production. (d) Production of enterotoxins and TSST-1. Data described in (Gottlieb et al. 2008).

Table 4.1 - Virulence assessment of the *S. aureus* strain collection. Tests include hemolytic activity, staphylokinase activity, catalase activity, carotenoid production and production of exotoxins. A sub-selection of strains were tested for their killing kinetics against *C. elegans* (time to 50% mortality, h) and their ability to survive and grow in human whole blood (cell density after 24 h, cfu/ml). From (Gottlieb et al. 2008).

Strain	Hemolysis	Staph.kin.	Catalase	Carotenoid	Enterotoxin				TSST-1	*C. elegans*	Blood
					A	B	C	D			
8325-4	+++	- [a]	++	+	-	-	-	-	-	184	2.4×10^8
Sa113	-	-	++	++	-	-	-	-	-		
Δ*mprF*	-	-	++(+)	++	-	-	-	-	-		
Δ*dltA*	-	-	+(+)	+	-	-	-	-	-		
14943	-	-	++(+)	+++	+++	-	-	-	+++	157	7.6×10^8
15033	++	-	+++	+++	-	+++	-	-	-		
B31369	++	-	++(+)	+++	-	+++	-	-	-	140	1.1×10^8
796	(+)	-	++(+)	+++	-	-	-	-	-	223	2.5×10^8
J15033	(+)	-	++	+++	-	-	-	-	-		
2148-jvi	-	-	++(+)	++	-	-	-	-	-	256	3.0×10^6
K3-B2	+	-	++	++	-	-	-	-	-	225	3.4×10^7
B29997	(+)	-	++(+)	+++	-	-	-	-	-		
KES 439	(+)	-	++	++	-	-	-	-	-		
KES 626	+++	- [a]	++(+)	+	-	-	-	-	-	118	1.0×10^9
KES 735	(+)	-	++	+++	-	-	-	-	-		
KES 855	++	-	++	++	-	-	-	-	-		

[a] A clearing zone on both plates with and without added serum indicate a high production of proteases.

There is considerably variation between the *S. aureus* strains in production of the single virulence factors tested in this study.

The strains differed markedly in hemolytic activity, reflecting their ability to lyse and kill phagocytic cells (Bhakdi et al. 1989;Foster 2005). In this study an animal clinical strain, 2148-jvi, was found to be non-hemolytic, while two human clinical strains, KES 439 and KES 735, were only weakly hemolytic, consistent with previous reports (Christensson and Hedstrom 1986;Clyne et al. 1988). We did not analyze the production of Panton-Valentine leukocidin, since this toxin is only found in a small subset of *S. aureus* strains (Prevost et al. 1995).

We did not detect any specific staphylokinase activity, although two strains (8325-4 and KES 626) showed a high level of unspecific proteolytic activity. This might reflect an increased ability to degrade host tissues, and indeed, KES 626 was isolated as a colonizer of a chronic wound (Gjødsbøl et al. 2006). Only three out of 143 *S. aureus* isolates from cows produced staphylokinase (Fitzgerald et al. 2000), while 66 out of 79 isolates from humans did (Humphreys et al. 1989). This is supported by the detection of anti-staphylokinase antibodies in all (120) samples of normal human sera, indicating that most people have been exposed to staphylokinase and emphasizing its importance in *S. aureus* infections *in vivo* (Rooijakkers et al. 2005a). In this context, it is odd that we did not detect any staphylokinase activity.

Also, the strains differed in their production of catalase and carotenoid, suggesting that the oxidative attack defense mechanisms contribute differently in each strain.

Strain 14943 (isolated from pork meat) produced both enterotoxin A and TSST-1, while 15033 and B31369 (isolated from pork meat and a clinical case) produced enterotoxin B. TSST-1 producing strains are often non-hemolytic (Christensson and Hedstrom 1986;Clyne et al. 1988), as is 14943. Enterotoxin A, B, and C production has been found to be higher among clinical isolates compared to nasal carriage isolates, suggesting a role for enterotoxins in diseases caused by *S. aureus* other than food poisoning (Humphreys et al. 1989).

Taken together, these analyses did not identify strains that were more or less virulent than the rest, suggesting that each parameter is important in different types of infection and that each strain is specialized in one (or more) type(s) of infection(s). However, it can be hypothesized that strains producing several virulence factors have higher virulence potential than strains producing a lower number of virulence factors. Comparing the presence of 33 putative virulence genes between invasive and carriage isolates of *S. aureus*, Peacock et al. found that seven genes (including *fnbA*, encoding fibronectin binding protein A, *cna*,

encoding collagen binding protein, and *hlg*, encoding gamma-toxin) were significantly more common in invasive isolates and that the effect of the seven virulence determinants seemed to be cumulative. This means that the more virulence determinants a strain possessed, the more likely it was to be an invasive strain as opposed to a carriage strain (Peacock et al. 2002). When we compared a sub-collection of the strains in two more complex virulence models - the nematode *C. elegans* and human whole blood - we found that the strains differed with respect to virulence against *C. elegans* but that only one strain, *S. aureus* 2148-jvi, differed noticeably from the others in growth in human whole blood (Figure 4.4) (Gottlieb et al. 2008).

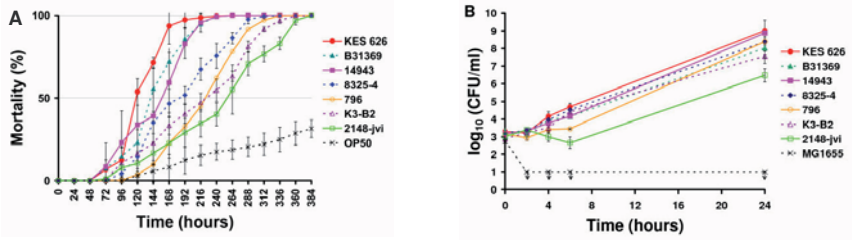


Figure 4.4: Virulence potential of *S. aureus* strains in *C. elegans* (a) and human whole blood (b). From (Gottlieb et al. 2008).

S. aureus 2148-jvi also showed the lowest virulence potential in *C. elegans* and had no hemolytic activity, suggestive of a lower virulence potential. However, this strain was isolated from a case of bovine mastitis, indicating that the poorer performance in human whole blood can potentially be related to species tropism (Hájek and Marsálek 1971;Devriese and Oeding 1976). The human clinical isolate, KES 626, killed *C. elegans* the fastest and grew well in human whole blood. It also had a high hemolytic and catalase activity, as well as a high unspecific proteolytic ability. On the other hand, it was unpigmented (Figure 4.3c), illustrating a low production of carotenoid. Others have found that the production of carotenoid contributed to staphylococcal survival in human whole blood due to its antioxidative potential (Liu et al. 2005). The discrepancy could be due to the use of different strains with different genetic backgrounds. It can be hypothesized that the carotenoid-attenuated field strain have had the opportunity to evolve other counter-protective measures or activate redundant antioxidative mechanisms compared to the laboratory-generated mutant of a carotenoid-producing wildtype, which would account for an increased survival in whole blood.

Although the determination of an array of virulence factors did not identify specific high- or low-virulent strains, it did confirm that the *S. aureus* collection used in this study represented a wide spectrum of virulence-associated phenotypes.

4.2. Listeria monocytogenes

4.2.1. General characteristics and natural niches

The genus *Listeria* currently includes six species, *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*. Only *L. monocytogenes* and *L. ivanovii* are pathogenic. *L. monocytogenes* is the primary cause of human listeriosis although rare cases have been caused by *L. ivanovii* that is otherwise mainly responsible for perinatal listeriosis in cattle and sheep. One human clinical case has been ascribed to the otherwise nonpathogenic *L. seeligeri* (Vazquez-Boland et al. 2001). Recently, a new species, *L. marthii*, closely related to *L. monocytogenes* and *L. innocua*, has been described. It has not been associated with disease to date (Graves et al. 2009).

L. monocytogenes is a small Gram-positive, non-sporeforming, motile, facultative anaerobic rod widely distributed in nature. It is found in both soil and water, with the primary habitat believed to be decaying plant matter including silage which can lead to infection of animals. In addition, both human and animal asymptomatic carriers have been described (Farber and Peterkin 1991;Iida et al. 1998;Vazquez-Boland et al. 2001). *L. monocytogenes* is able to grow under adverse conditions such as temperatures between 0 – 44°C, at salt concentrations as high as 10%, and at pH intervals from 4.4 up to 9.6 (Dykes and Moorhead 2000).

For epidemiological purposes, *L. monocytogenes* is divided into subgroups based on phenotypic and genotypic traits. The presence of a combination of somatic (O) and flagellar (H) surface proteins on *L. monocytogenes* strains divide them into 13 serogroups: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7 (Seeliger and Höhne 1979). Intriguingly, more than 90% of human clinical cases are caused by serotype 1/2a, 1/2b or 4b (Farber and Peterkin 1991;Vazquez-Boland et al. 2001). Most cases (64%) of listeriosis are due to serotype 4b, while serotype 1/2a and serotype 1/2b only cause 15% and 10%, respectively. Serotype 1/2c is responsible for 4% of cases (McLauchlin 1990). As serotypes 1/2a, 1/2b, and 1/2c predominate in foods (Farber and Peterkin 1991;Vazquez-Boland et al. 2001), this suggests that some serotypes, especially 4b, are more virulent than others (McLauchlin 1990).

Sequence differences in several central virulence genes divide *L. monocytogenes* into three distinct evolutionary lines that correlate with flagellar antigens (Rasmussen et al. 1995;Wiedmann et al. 1997). Strains from human cases are restricted to lineage I and II, while strains from animal cases are distributed among all three lineages. Lineage I contains strains from human epidemic outbreaks, whereas linage II only contains sporadic cases. Lineage I contains strains belonging to serotype b, and lineage II contains serotypes a and c. Also, strains from lineage I and III showed higher virulence potential than strains from lineage II. This suggested that linage I and III contain strains that are highly virulent to humans and animals, while lineage II contains strains with lower virulence potential (Wiedmann et al. 1997). When we tested the tolerance of *L. monocytogenes* strains to HDPs and H_2O_2, we found that lineage II strains were more sensitive to novicidin, while lineage I strains were more susceptible to plectasin. However, when the tolerance was compared to the four HDPs as a group or to H_2O_2, no differences between lineages were observed (Table 3.1) (Gottlieb et al. 2008).

Since the majority of human-associated *L. monocytogenes* strains belong to only three serotypes within one lineage, several molecular subtyping methods have been employed to further sub-group isolates. The strains used in this study have been subtyped using randomly amplified polymorphic DNA (RAPD) typing. This method is based on random amplification of DNA by short, arbitrary PCR primers under low-stringent annealing conditions. The advantage of this method is that it is a rapid, low cost typing approach that is reproducible and as discriminatory as pulsed field gel electrophoresis (PFGE) when strict standardization is exhibited, especially with respect to purification of target DNA (Vogel et al. 2001a).

4.2.2. Routes of contamination and infection

Reports of Gram-positive rods from tissues of diseased patients, which in retrospect probably suffered from listeric infections, date back to 1891 as reviewed in (Gray and Killinger 1966). However, *L. monocytogenes* was first described under the name *Bacterium monocytogenes* as the causative agent of mononucleosis in rabbits and guinea pigs by Murray and co-workers in 1926 (Murray et al. 1926) and in 1940 Pirie suggested to change the genus name to *Listeria* (Pirie 1940). The first confirmed human case was described in Denmark in 1929 (Nyfeldt 1929). However, it was not until an outbreak in Nova Scotia in 1981 that *L. monocytogenes* was recognized as a foodborne pathogen (Schlech et al. 1983).

L. monocytogenes is introduced to the food processing environment either with contaminated raw material (of both animal and vegetal origin), soil, or possibly also by healthy human carriers. Once introduced, specific subtypes of *L. monocytogenes* have been shown to

persist in the food processing environment for long periods of time despite comprehensive cleaning and disinfectant procedures (Rørvik et al. 1995;Autio et al. 1999;Norton et al. 2001;Vogel et al. 2001a;Wulff et al. 2006). Thus, food products can become contaminated, potentially giving rise to food borne infections (Olsen et al. 2005b). This emphasizes the need for a thorough understanding of the physiology and the virulence potential of such persistent subtypes. The assessment of virulence potential is further discussed in section 4.2.4 and in chapter 5.

Listeriosis occurs mainly in two forms: Either as a mild, gastrointestinal illness or as a serious invasive disease with a high mortality. The clinical outcome of *L. monocytogenes* infection depends on the number of ingested bacteria, the virulence potential of the bacteria and the immune status of the host (Vazquez-Boland et al. 2001). In healthy, immunocompetent adults, ingestion of low doses of *L. monocytogenes* will probably have no effect except for possible development of anti-listerial immunity. On the other hand, ingestion of heavily contaminated foods is likely to cause typical gastroenteritis symptoms including diarrhea, vomiting and fever. It occurs 18-20 hours after ingestion, and the infectious dose is estimated to be high (up to 10^9 CFU). The gastrointestinal variant is self-limiting and normally resolves spontaneously without any permanent damage (Vazquez-Boland et al. 2001;Lecuit 2007).

The invasive form of listeriosis most often targets immonucompromised individuals, i.e. the elderly, pregnant women, their fetus, or newborns. The intestinal tract is the major site of entry, and after crossing the intestinal barrier, *L. monocytogenes* disseminates in the blood to the primary target organs, the liver and spleen, where it is normally eliminated by resident macrophages (Figure 4.5).

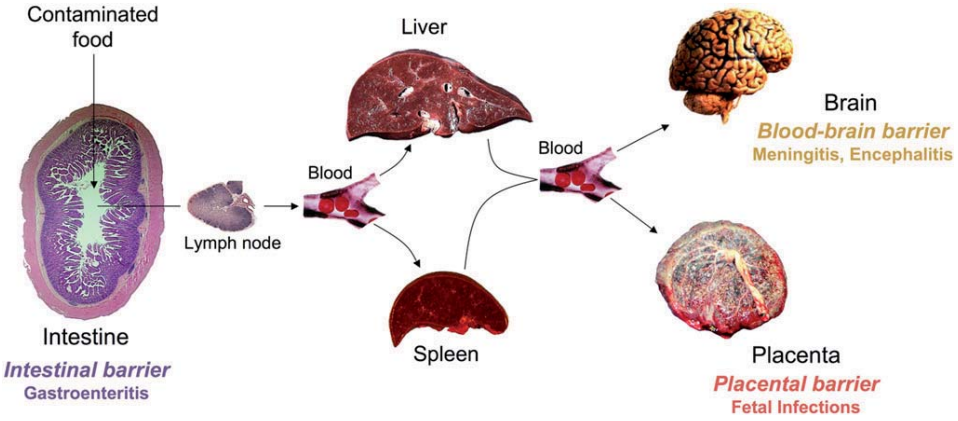


Figure 4.5: Course of infection of human listeriosis. From (Lecuit 2007).

Failure to mount a proper immune response in immunocompromised individuals result in unrestricted growth of *L. monocytogenes* until it reaches a critical mass and is released to the bloodstream. The ensuing bacteremia results in local infections in the secondary target organs, the brain (causing meningitits and encephalitis) and the placenta, or in septicemia. In pregnant women, the infected mother often only gets mild flu-like symptoms, whereas the fetus is infected by transplacental transmission of *L. monocytogenes* from the maternal blood which cause abortion, stillbirth, or a generalized infection of the neonate known as granulomatosis infantiseptica (Farber and Peterkin 1991;Vazquez-Boland et al. 2001;Roberts and Wiedmann 2003). The treatment of choice for listeriosis is co-administration of penicillin G or ampicillin with an aminoglycoside, classically gentamicin (Charpentier and Courvalin 1999). Even with antibiotic treatment, listeriosis has a mortality rate as high as 30% (Vazquez-Boland et al. 2001;Roberts and Wiedmann 2003). The prolonged incubation period before clinical manifestation of invasive disease could be due to both a period of silent intracellular replication in the hepatocytes as well as asymptomatic bacteremia.

4.2.3. Virulence factors in *L. monocytogenes*

L. monocytogenes is an intracellular pathogen that is able to induce its own internalization into non-phagocytic cells including epithelial cells, fibroblasts, hepatocytes, endothelial cells, and neurons (Vazquez-Boland et al. 2001). The strategy of adapting an intracellular life style gives it the advantage of “hiding” inside host cells, thus escaping the humoral immune system. The intracellular life cycle starts with the active uptake of *L. monocytogenes* mediated by bacterial surface proteins called internalins (Figure 4.6).

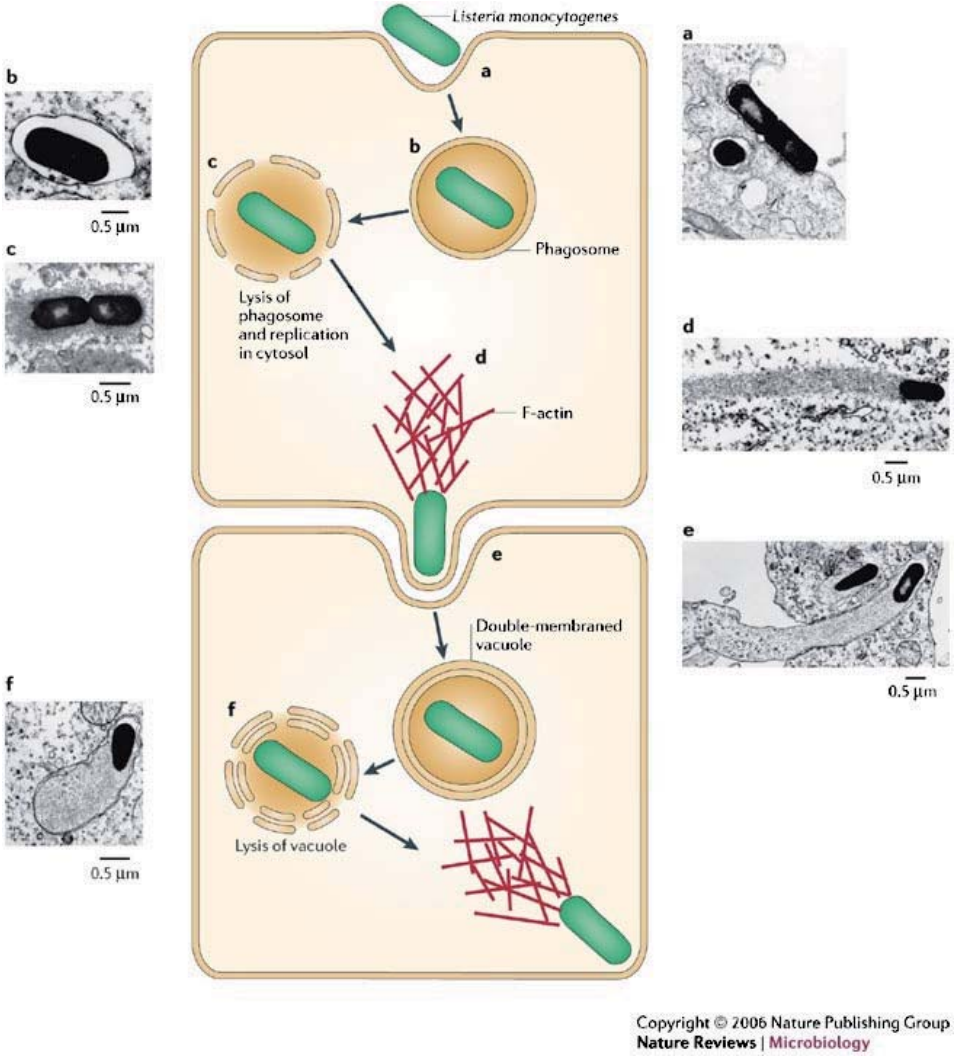


Figure 4.6: The intracellular life cycle of *L. monocytogenes*. (a) Entry of *L. monocytogenes* into non-phagocytic cells is an active process induced by the surface proteins InlA and InlB. Lysis from the vacuole and escape to the cytosol is mediated by the secretion of the hemolysin listeriolysin O, LLO, and a phospholipase, PI-PLC. Upon intracellular replication, *L. monocytogenes* is able to recruit the host cell actin-polymerization machinery via the action of ActA and propel itself from one cell to the neighboring cells. This results in a double-membraned vacuole that is lysed due to the action of LLO and another phospholipase, PC-PLC, and the intracellular cycle continues. See text for details. From (Hamon et al. 2006).

Internalin A mediates the invasion into epithelial cells (Gaillard et al. 1991) via the interaction with the E-cadherin receptor (Mengaud et al. 1996). InlB mediates the invasion into other cells (Dramsi et al. 1995) via the Met receptor (Shen et al. 2000) or the complement receptor gC1q-R (Braun et al. 2000). *L. monocytogenes* attaches to and is surrounded by the host cell membrane, and internalization results in the engulfment of *L. monocytogenes* in a vacuole (Figure 4.6b). As in phagocytes, the vacuole acidifies; thus survival of *L. monocytogenes* is dependent on its ability to escape being mediated by the hemolysin Listeriolysin O (LLO) (Portnoy et al. 1988;Cossart et al. 1989). Two phospholipases, phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase C (PC-PLC), are also secreted by *L. monocytogenes* and participate in the escape from the vacuole (Geoffroy et al. 1991;Mengaud et al. 1991a). Following the escape, *L. monocytogenes* replicates in the cytosol. Doubling times have been estimated as being between 40 minutes and one hour (Vazquez-Boland et al. 2001;Portnoy et al. 2002). Intracytoplasmic *L. monocytogenes* are immediately surrounded by actin filaments which are recruited to one of the poles of the bacterium by the action of the surface protein ActinA (Tilney and Portnoy 1989;Kocks et al. 1992). Here they are polymerized to form an actin-tail that propels *L. monocytogenes* through the cytoplasm in a random manner. Eventually, *L. monocytogenes* reaches the periphery of the cell and is pushed into the neighboring cell where it is engulfed by the recipient host cell membrane and internalized, resulting in a double-membrane vacuole. *L. monocytogenes* escapes from this vacuole within 5 minutes and initiates a new cycle of intracellular proliferation and intracellular spread (Vazquez-Boland et al. 2001).

The above mentioned virulence genes are clustered together in two loci in the genome known as pathogenicity islands. The genes for LLO (*hly*), PI-PLC (*plcA*), PC-PLC (*plcB*) and ActA (*actA*) are physically linked in the virulence gene cluster Listeria pathogenicity Island 1 (LIPI-1) (Vazquez-Boland et al. 2001). The genes encoding InlA (*inlA*) and InlB (*inlB*) are located in an internalin island in another part of the chromosome (Vazquez-Boland et al. 2001). The virulence genes in LIPI-1 are under the control of the transcriptional regulator, positive regulatory factor A (PrfA), encoded by *prfA* which is itself located in LIPI-1 (Mengaud et al. 1991b). PrfA is also involved in the regulation of *inlA* and *inlB* expression. *prfA* is thermoregulated at the posttranscriptional level, as *prfA* mRNA forms secondary structures at 30°C that resolves at 37°C, allowing for the translation of *prfA* and the subsequent expression of virulence genes when inside the host (Johansson et al. 2002).

4.2.4. Assessment of virulence potential

Several studies have indicated that *L. monocytogenes* strains differ in their virulence potential (Chakraborty et al. 1994;Sokolovic et al. 1996;Norrung and Andersen 2000;Buncic et al. 2001;Roche et al. 2003), and some of these differences may be explained by polymorphisms in virulence genes. The underlying genetic mechanisms that may be responsible for differences in virulence among subtypes of *L. monocytogenes* can be addressed by sequencing the responsible genes. Epidemiological studies of the expression of central virulence genes, including *inlA, inlB*, *hly*, and *actA*, in two *L. monocytogenes* populations of clinical and food origin identified the presence of a full length InlA as a marker for virulence, since this was present in 96% of clinical *L. monocytogenes* isolates (a group with a priori high virulence potential) compared to only 65% of *L. monocytogenes* isolates from food, a group that might contain strains with low or attenuated virulence (Jacquet et al. 2002;Jacquet et al. 2004). No polymorphisms were described in InlB or LLO, but the strains could be divided into four groups based on the molecular weight of ActA (Jacquet et al. 2002).

The reference strain *L. monocytogenes* LO28 harbors a nonsense mutation in *inlA* which results in a truncated InlA that is released to the medium and consequently, LO28 show decreased invasive ability (Jonquieres et al. 1998). Several other studies have identified field strains with single point mutations causing premature stop codons in *inlA*, and correlated the expression of a truncated InlA to attenuated virulence (Olier et al. 2002;Olier et al. 2003;Nightingale et al. 2005;Temoin et al. 2008;Nightingale et al. 2008). Hence, a full length InlA has been suggested as a potential biomarker to assess the virulence potential of *L. monocytogenes* strains (Jacquet et al. 2004). We have assessed the virulence potential of a group of *L. monocytogenes* strains of different origins and found that four very similar *L. monocytogenes* strains that all grouped in a particular RAPD group (denoted type 9) all encode an *inlA* with a premature stopcodon (Figure 4.7) (Holch et al. 2009). The four RAPD type 9 strains represent a sub-type that is often persistent and dominant in the food industry (Wulff et al. 2006). Interestingly, the stop codon is at the same position as the one described by Olier et al. (Olier et al. 2002;Olier et al. 2003). The strains harboring this mutation also showed decreased virulence in Caco-2 cells (Jensen et al. 2007a) and human trophoblastic cells (Holch et al. 2009) as is further discussed in chapter 5.

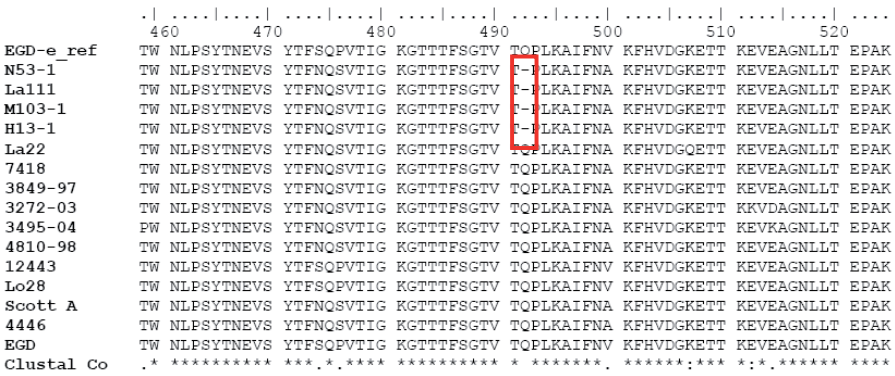


Figure 4.7: Sequence of *inlA* from the *L. monocytogenes* strains used in this study. The four persistent RAPD type 9 strains all harbor a nucleotide substitution resulting in a stopcodon at position 492 and the expression of a truncated InlA. Adapted from (Holch et al. 2009).

However, the occurrence of truncated InlA have also been described in strains of clinical origin (Jonquieres et al. 1998;Jacquet et al. 2004) emphasizing that even though a full length InlA can be a biomarker for virulence (Jacquet et al. 2004), the opposite, a truncated InlA, is not necessarily a biomarker for decreased or attenuated virulence. We have also identified the presence of a 9 bp deletion in *inlA* in a strain of fetomaternal origin (*L. monocytogenes* 4810-98) (Holch et al. 2009). This deletion has also been described in two serotype 4b strains isolated from cod roe, and in accordance with our observations it did not affect the invasion into Caco-2 cells (Handa-Miya et al. 2007).

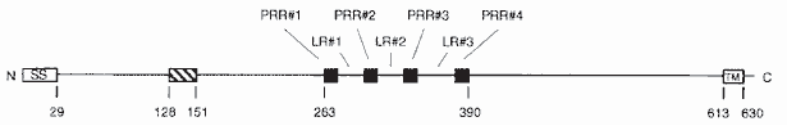
The invasive ability is not completely abolished in the RAPD type 9 strains despite a truncated InlA, indicating that other, although less efficient ligand-receptor interactions, are involved in *L. monocytogenes* internalization. One such ligand could be the Listeria adhesion protein (LAP) that interacts with Heat shock protein 60 (Hsp60) and also mediates internalization of *L. monocytogenes* into Caco-2 cells (Wampler et al. 2004).

In contrast to the epidemiological studies that did not detect polymorphisms in *inlB*, another study showed that a group of low-virulence strains of food origin harbored several point mutations in *inlB*, some of these causing amino acid substitutions in the region interacting with the Met receptor (Temoin et al. 2008). This resulted in lower invasive ability in Vero fibroblastic cells. Interestingly, we found the same point mutation in the four RAPD type 9 strains as well as in a number of other strains, but did not observe an effect of the mutation on the invasive ability in L929 fibroblastic cells (Holch et al. 2009). The discrepancy could be due to the use of two different cell lines in the two studies (Vero cells are derived from the

green monkey, whereas L929 cells are of murine origin) or that other factors in these strains influence the invasion.

The differences in molecular weight of ActA observed in the epidemiological study (Jacquet et al. 2002) are related to allelic variation. ActA can be divided into three functional domains (Figure 4.8a) including a membrane-anchoring C-terminal domain, a central domain containing three to four proline-rich repeats (PRR), and an N-terminal domain essential for stimulating actin polymerization (Kocks et al. 1992;Smith et al. 1996;Skoble et al. 2000). Several studies have observed allelic variation in the number of PRRs in *L. monocytogenes* strains (Sokolovic et al. 1996;Smith et al. 1996;Wiedmann et al. 1997;Moriishi et al. 1998;Jiang et al. 2006;Roberts and Wiedmann 2006;Holch et al. 2009) causing *actA* transcripts or ActA proteins of different sizes. The repeat motifs have been demonstrated to enhance actin-based motility with movement rates being proportional to the number of PRRs (Smith et al. 1996). Consequently the number of repeats has been related to plaque forming ability and virulence, although with contradicting results.

a)



b)

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                 | ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 290        300        310        320        330        340        350        360
EGD-e_ref        N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIIR ETASSLDSSF
N53-1            N APTPSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSSF
La111            N APTPSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSSF
M103-1           N APTPSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSSF
H13-1            N APTPSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSSF
La22             N APTTSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSSF
7418             N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSSF
3849-97          N APATSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSSF
3272-03          N APTPSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSSF
3495-04          N APATSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSSF
4810-98          N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSSF
12443            N APATSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSSF
Lo28             N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIIR ETASSLDSSF
Scott A          N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSSF
4446             N APATSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSSF
EGD              N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIIR ETASSLDSSF
Clustal Co       * **:.****** *****                                        ********:* ***.******
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c)

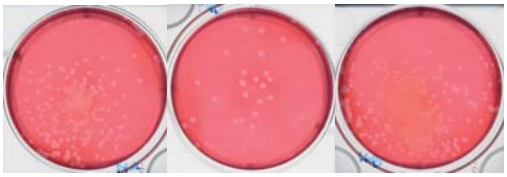

Figure 4.8: Schematic representation of ActA and variation in *actA*. (a) Schematic representation of ActA with the central PRRs. From (Smith et al. 1996). (b) Sequence of *actA*. Eight strains have a 105 nt deletion, corresponding to one proline-rich-repeat. (c) Plaque forming by *L. monocytogenes* La111, EGD and 7418 (from left to right). From (Holch et al. 2009).

A *L. monocytogenes* strain expressing a truncated ActA (3 PRRs) compared to the *L. monocytogenes* EGD ActA (four PRRs) had attenuated virulence in mice (Chakraborty et al. 1994). Likewise, Sokolovic et al. found that the lower virulence phenotype of a serotype 4a strain compared to 4b strains correlated with three PRRs and a smaller plaque size (Sokolovic et al. 1996). Accordingly, Jiang et al. have described a strain carrying three PRRs that was unable to form plaques compared to another strain carrying four PRRs (Jiang et al. 2006). In contrast, *L. monocytogenes* isolates belonging to lineage I predominantly carried three PRRs and showed larger plaque sizes than lineage II isolates predominantly carrying four PRRs, suggesting that the presence of three PRRs is linked to higher virulence (Wiedmann et al. 1997). Other studies could not correlate the number of PRR to neither serotype (Moriishi et al. 1998) nor plaque forming ability (Roberts and Wiedmann 2006). In agreement with these studies and in contrast to Wiedmann et al. (1997), we could not correlate the number of PRRs with serotype, plaque forming ability, or lineage (Figure 4.8b and 4.8c) (Holch et al. 2009). The difference in plaque forming ability observed by Jiang et al. (Jiang et al. 2006) is probably due to the comparison of two strains with different genetic backgrounds. Roberts and Wiedmann specifically tested the importance of genetic background using allelic-exchange mutagenesis, showing that the plaque forming ability was not dependent on the number of PRRs although the number of PRRs could contribute to the attenuated virulence observed in some strains carrying only three PRRs (Roberts and Wiedmann 2006).

4.3. Conclusions

S. aureus and *L. monocytogenes* are two related Gram-positive pathogenic bacteria that represent different natural niches and routes of infection. The *S. aureus* strains used in this study differ in their production of an array of virulence factors. However, single strains cannot be identified as more or less virulent than the rest, suggesting that each virulence factor contribute to different types of infection and that each strain is specialized in one (or more) type(s) of infection(s). The *L. monocytogenes* RAPD type 9 strains harbor a premature stop codon leading to a truncated *inlA* that probably accounts for the lower virulence potential of these strains. Eight of the 15 strains tested in the study had a 105 bp deletion in *actA*. This deletion is in the proline rich repeat region and does not seem to affect the virulence potential of these strains.

5. Eukaryotic cells as models for virulence assessment in *L. monocytogenes*

Several laboratories, including ours, have demonstrated that a food processing unit can harbor particular molecular sub-types for long periods of time (Rørvik et al. 1995;Norton et al. 2001;Wulff et al. 2006). These strains are likely contaminants of food products and hence a potential health risk to consumers. At the same time, the virulence of *L. monocytogenes* strains differ greatly, and several reports exist on the widespread presence of *L. monocytogenes* strains with attenuated virulence in foods (Jacquet et al. 2004;Nightingale et al. 2005). If the *L. monocytogenes* persisting in the food industry are indeed low virulent, one could speculate that they might serve to keep more virulent strains from entering the food processing plant. Understanding and assessing the virulence potential of *L. monocytogenes* strains will help to assess the real risk posed by different sub-populations of *L. monocytogenes* in foods. Subsequently, one could imagine that different criteria may be employed for regulation of *L. monocytogenes* in foods depending on the virulence of the organism.

The true virulence of *L. monocytogenes* can, however, only be determined in a natural model of listeriosis, since the potential severity of *L. monocytogenes* infection rule out the use of human volunteers. Instead, various animal models including mice (Lecuit et al. 2001;Czuprynski et al. 2003;Kim et al. 2004), guinea pigs (Bakardjiev et al. 2004;Andersen et al. 2007;Jensen et al. 2008a;Jensen et al. 2008b), gerbils (Disson et al. 2008;Disson et al. 2009), and non-human primates (Farber et al. 1991;Smith et al. 2003) have been applied to assess the virulence potential. However, due to ethical concerns and restrictions, as well as the costs and workload associated with such experiments, *in vivo* animal models are not appropriate for screening of large collections of field strains. As an alternative, various *in vitro* cell assays have been designed that exploit the capability of *L. monocytogenes* to adhere, invade, grow inside and spread between various mammalian cells. These assays use established mammalian cell lines to mimic the stage (or phase) specific barriers that *L. monocytogenes* crosses during infection, and focus on assessing the active invasion into non-phagocytic cells and the intracellular cell-to-cell spread in both epithelial and fibroblastic cells (Figure 5.1) (Liu et al. 2007). In addition to the *in vitro* cell lines, several non-mammalian animals have been used for virulence assessment of *L. monocytogenes* such as the nematode *Caenorhabditis elegans* (Thomsen et al. 2006;Forrester et al. 2007) and the fruitfly *Drosophila melanogaster* (Mansfield et al. 2003;Jensen et al. 2007b).

We have previously shown that one molecular sub-type of *L. monocytogenes* is persisting in several fish processing industries. The *L. monocytogenes* strains were sub-typed using randomly amplified polymorphic DNA profiles, and this particular group denoted RAPD type 9 (Wulff et al. 2006). These strains appear to have a low virulence potential when assessed in human intestinal cells, in the non-mammalian hosts *C. elegans*, and *D. melanogaster* and in non-pregnant guinea pigs (Jensen et al. 2007a;Jensen et al. 2008a). However, when assessing the virulence potential in a more complex model (a pregnant guinea pig), one of these RAPD type 9 strains, La111, invaded the guinea pig fetuses to the same extent as the monkey clinical strain 12443 and better than Scott A (Jensen et al. 2008b). We hypothesized that this paradox reflected a specific ability of the RAPD type 9 strains to execute one (or several) of the specific steps involved when *L. monocytogenes* crosses the placenta membrane. We therefore compared the adhesion and invasion of the RAPD type 9 strains and a collection of other *L. monocytogenes* strains in several cell line models (Figure 5.1) (Holch et al. 2009). We reasoned that the phenotypic behavior of the RAPD type 9 strains in these cell models would be similar to strains that are known to have caused fetal infection.

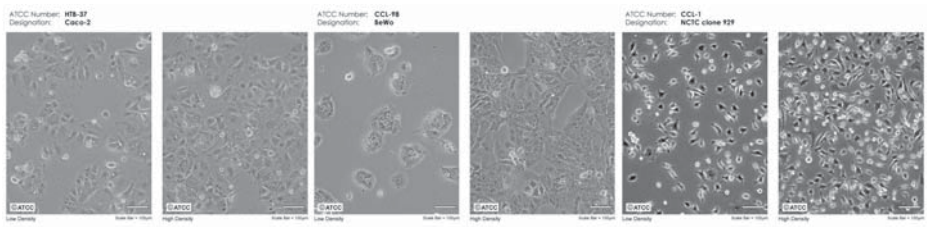

Figure 5.1: Microscopic appearance of the cell types used in this study. Left: Caco-2 intestinal, epithelial cells. Middle: BeWo trophoblastic, epithelial cells. Right: L929 fibroblastic cells. Scale bar 100 µm. From (http://www.lgcstandards-atcc.org/Attachments/1777.jpg).

5.1. Intestinal barrier

Being a food borne pathogen, *L. monocytogenes* first encounters and interacts with epithelial cells of the mammalian gastrointestinal tract during infection. *L. monocytogenes* has a unique ability to actively invade non-phagocytic cells and hence can cross the three tight physiological barriers of the host, the gastro-intestinal barrier, the blood-brain barrier and the placental barrier. *In vivo* studies of *L. monocytogenes* infection have shown that intestinal invasion occurs in the small intestine as well as the caecum and colon (Disson et al. 2008). The human colonic cancer epithelial cell line Caco-2 was established as a model of infection of intestinal epithelial cells by *L. monocytogenes* and demonstrated that unlike the non-

pathogenic species (*L. seeligeri*, *L. welshimeri* and *L. innocua*), the pathogenic *L. monocytogenes* and *L. ivanovii* were able to enter Caco-2 cells by inducing their own phagocytosis (Gaillard et al. 1987). In line with this study, Pine et al. used the cytopathogenic effects in infected Caco-2 cells to discriminate between virulent and nonvirulent *L. monocytogenes* strains as determined by lethal doses (LD_{50}) in mice (Pine et al. 1991). The invasive ability in Caco-2 cells has also been correlated to serotype, with strains of all 13 serotypes being divided into two groups of high and low invasive ability, respectively. The majority of the serogroup 4b, 1/2a, and 1/2b strains (12 out of 14 strains) grouped into the high invasive group (Jaradat and Bhunia 2003) further indicating the significance of invasion into Caco-2 cells as a measure of virulence potential. Likewise, Larsen et al. showed that *L. monocytogenes* strains of different PFGE types had significantly different invasive abilities. A group of PFGE type 1 strains had invasion at log 2.5 CFU per well compared to a group of high invasion with an average of 3.5 CFU per well (Larsen et al. 2002). However, as different studies compute the invasion differently (e.g. either directly as the number of recovered intracellular bacteria, as the intracellular bacteria relative to the original inoculum, or as the number of intracellular bacteria relative to the number of adherent and invaded bacteria), comparison between experiments is difficult. In addition, the invasive efficiency also varies with the multiplicity of infection (MOI), i.e. the number of bacteria added per cell in the monolayer. A higher invasion efficiency is observed at low MOI (Francis and Thomas 1996), which further complicates comparisons.

Another human colonic cancer cell line (HT-29) (Roche et al. 2001) as well as the small intestine cell line INT-407 (Jaradat et al. 2003) have also been used to assess the virulence potential of *L. monocytogenes* strains. Caco-2 cells have been shown to have an unusually high ability to internalize *L. monocytogenes*. The average number of invaded bacteria in Caco-2 cells was 97% of the original inoculum compared to 0.1 to 10% for other cell lines, including INT-407 (Pine et al. 1991). Also, the cell immortalization (i.e. cell lines) as opposed to primary cell cultures enhances *L. monocytogenes* invasion since proliferative, undifferentiated cells were more susceptible to entry of *L. monocytogenes* than non-proliferative, differentiated cells (Velge et al. 1994;Velge et al. 1997). Accordingly, other studies have used the HT-29 cell line instead of Caco-2 cells since the presence of glucose in the medium maintains the HT-29 cells at a constant proliferation rate (Roche et al. 2001). These results suggest that invasion studies in Caco-2 cells might overestimate the invasive ability of the strains tested.

Nevertheless, Caco-2 cells is a widespread model to assess the ability of *L. monocytogenes* strains to adhere, invade and replicate inside enterocytes (Larsen et al. 2002;Jaradat and

Bhunia 2003;Wampler et al. 2004;Rousseaux et al. 2004;Andersen et al. 2006;Yamada et al. 2006;Jensen et al. 2007a;Holch et al. 2009).

The invasion of *L. monocytogenes* into Caco-2 cells is InlA-dependent (Mengaud et al. 1996) and consequently, *L. monocytogenes* LO28 as well as other strains encoding a truncated InlA have lower invasion into Caco-2 cells compared to strains expressing a full length InlA (Jonquieres et al. 1998;Olier et al. 2002;Olier et al. 2003;Nightingale et al. 2005;Felicio et al. 2007).

We have previously shown that the specific molecular sub-type (RAPD type 9 strains) are poor invaders of Caco-2 cells, invading in cell numbers that are 100-1000 fold lower than seen with typical clinical strains (Jensen et al. 2007a;Jensen et al. 2008a). Since we hypothesized that the RAPD type 9 strains would behave similar to strains of fetomaternal origin in cell virulence models, we determined the invasive ability of a group of maternofetal strains and found that they invaded Caco-2 cells in the same high level as other clinical strains (Figure 5.2) (Holch et al. 2009).

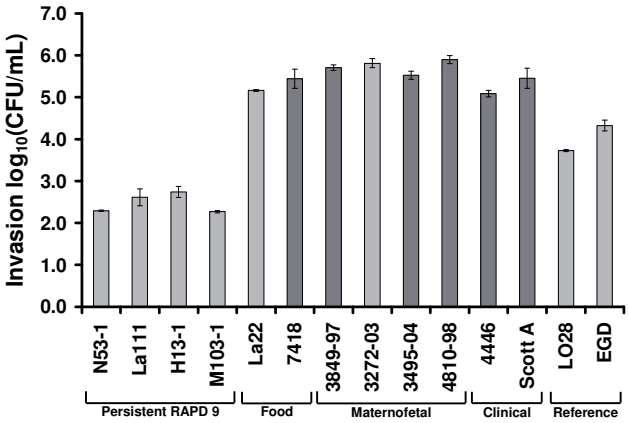

Figure 5.2: Caco-2 cell invasion of *L. monocytogenes* strains used in this study. Strains have been sorted according to origin. Dark columns represent lineage I strains and light columns represent lineage II strains. Columns represent averages from one trial carried out in duplicate and error bars are standard deviations. The results are representative of three independent experiments. Compilation of data from (Jensen et al. 2007a;Holch et al. 2009).

We found that the RAPD type 9 strains all carried a specific nonsense mutation in *inlA* (Figure 4.7) and this most likely account for the lower invasion of these strains in Caco-2 cells (Holch et al. 2009). In comparison, field strains harboring another premature stop codon

(at position 25) invaded Caco-2 cells on average 55-fold less than the EGDe wildtype (Temoin et al. 2008).

Interestingly, the occurrence of field stains with nonsense mutations in *inlA* and consequently low invasive ability in Caco-2 cells seem to be widespread. It has been described in strains from France (Rousseaux et al. 2004;Temoin et al. 2008), the United States (Nightingale et al. 2005;Nightingale et al. 2008), Portugal (Felicio et al. 2007), and Japan (Handa-Miya et al. 2007). These virulence-attenuated strains are most commonly found among lineage II serotype 1/2a and 1/2c strains, and the prevalence in foods is as high as 30% (Jacquet et al. 2004;Nightingale et al. 2005) although the Japanese study only identified one strain carrying an *inlA* PMSC out of 59 tested food strains (Handa-Miya et al. 2007). We have identified an *inlA* PMSC in one strain originally isolated from cold-smoked salmon (La111) (Vogel et al. 2001a) and in three strains (N53-1, M103-1, and H13-1) that were all isolated from fish processing plants (Wulff et al. 2006) and thus potentially could contaminate food but the general prevalence of *inlA* PMSCs among Danish *L. monocytogenes* food isolates is not known.

It has been suggested that the rather high prevalence in food and hence the frequent ingestion of such virulence-attenuated *L. monocytogenes* strains might result in asymptomatic carriage and a mucosal immune response conferring resistance in humans to infection by *L. monocytogenes*. Indeed, vaccination of guinea pigs with a virulence attenuated strain carrying an *inlA* PMSC reduced the severity of subsequent infection with a fully virulent strain (Nightingale et al. 2008). However, Iida et al. observed that among carriage strains isolated from a healthy Japanese population, 94.7% belonged to serotype 1/2a, 1/2b or 4b, suggesting that if persons in high risk groups (such as pregnant women) become carriers, they could have a high risk of developing listeriosis (Iida et al. 1998).

5.2. Blood-placenta barrier

During pregnancy, the fetus can immunologically be regarded as an allograft and maternal tolerance to the paternally derived fetal antigens is thought to occur through local suppression of cell-mediated immunity at the maternofetal interface (the placenta). The prevailing hypothesis is that pregnancy-related hormonal factors as well as cytokines released by maternal macrophages and fetal trophoblasts at the maternofetal interface affect the T_H1-T_H2 balance towards a T_H2-dominated humoral immunity and a suppression of cell-mediated immunity (Guleria and Pollard 2000;Dealtry et al. 2000;Abram et al. 2003;Jamieson et al. 2006). This increases the susceptibility of pregnant women to intracellular pathogens such as *L. monocytogenes*, *Toxoplasma gondii*, and cytomegalovirus (Ross et al.

2006;Jamieson et al. 2006). *L. monocytogenes* is capable of crossing the placental barrier, and it has been proposed that the placenta offers an immunologically privileged compartment within which *L. monocytogenes* can replicate and eventually spread to the fetus (Redline and Lu 1987;Redline and Lu 1988;Abram et al. 2003;Le Monnier et al. 2006;Bakardjiev et al. 2006). We speculated that the otherwise low-virulent RAPD type 9 strains might be opportunistic pathogens, conferring protection in a healthy population but posing a particular risk to pregnant women due to the T_H1-T_H2 shift occurring during pregnancy.

The placenta is composed of interlocking maternal and fetal tissues and is formed upon implantation when fetal epithelial cells, trophoblasts, proliferate and form protrusions into the uterus wall thereby forming the placental villi (Leiser and Kaufmann 1994). During the invasion of the uterus endometrium, the fetal trophoblasts erode the walls of maternal capillaries, ensuring an adequate blood supply to the intervillous space through which the exchange of nutrients and waste products between the maternal and fetal blood streams occur (Figure 5.3).

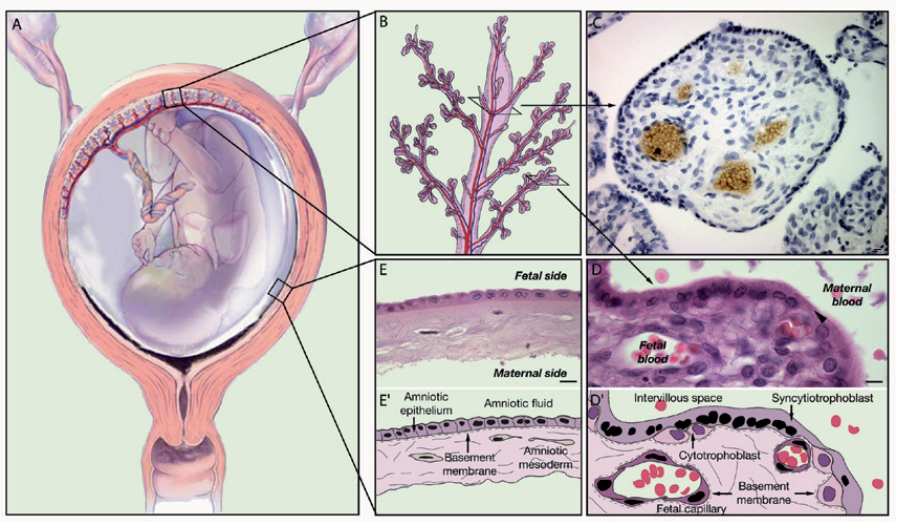

Figure 5.3: The anatomy of the maternofetal barrier in humans. (A) The fetus in the uterine cavity. (B) The placental villus tree. (C-D) Cross section of a villus. Nutrients, waste products and also some drugs and infectious agents are exchanged between the maternal blood in the intervillous space and the fetal blood. The placental membrane is thus a semipermeable layer of fetal tissues that separates the maternal and fetal blood streams. It is composed of the trophoblasts covering the villi, the connective tissue of the villi and the endothelial cells lining the fetal vessels. (E) Cross section of the amnion that delineates the extraplacental maternofetal interface. From (Lecuit et al. 2004).

After invasion of the gastrointestinal epithelium and successful replication in the liver, *L. monocytogenes* disseminates in the blood to its secondary target organs, the brain and the placenta. Thus, to infect the fetus, *L. monocytogenes* from the maternal blood must first invade the fetal trophoblasts and spread through the connective tissue of the villi before it can cross the endothelial cells of the fetal blood vessels and gain access to the fetal bloodstream (Figure 5.3d). The mechanisms responsible for the vertical transmission of *L. monocytogenes* across the fetoplacental barrier are not fully understood. Cell line-based studies have focused on the invasion into trophoblasts, as they have been shown to be the first placental cell type that *L. monocytogenes* interacts with during *in vivo* placental infection (Lecuit et al. 2004;Bakardjiev et al. 2004;Le Monnier et al. 2006).

5.2.1. Invasion of trophoblasts

A number of different cell lines have been used to study the interaction of *L. monocytogenes* and trophoblasts, including the choriocarcinoma cell lines BeWo, JAR, and JEG-3 (Lecuit et al. 2004;Bakardjiev et al. 2004;Disson et al. 2008;Mostowy et al. 2009). These studies have primarily focused on the differences in invasive ability between *L. monocytogenes* wildtype and *inlA* and *inlB* mutants. *In vitro* studies in the BeWo cell line have shown that *L. monocytogenes* invades trophoblasts in an InlA-dependent manner. Whereas *inlB* mutants did not differ from the parental wildtype with respect to invasion of trophoblasts, invasion of the wildtype was approximately 20-fold (Lecuit et al. 2004) and 100-fold (Bakardjiev et al. 2004) greater than the isogenic *inlA* mutant. In contrast, both InlA and InlB are involved in the invasion of *L. monocytogenes* into JAR cells, with a 10-fold difference in invasive efficiency between *inlA* or *inlB* mutants and their isogenic wildtype (Disson et al. 2008). Also, *L. monocytogenes* did not show enhanced invasion into the BeWo cell line compared to primary cultured trophoblasts (Lecuit et al. 2004;Bakardjiev et al. 2004), suggesting that in contrast to Caco-2 cells (Velge et al. 1994) immortalization does not influence the susceptibility of BeWo cells to *L. monocytogenes* infection.

We have used the JAR cell line to assess the invasive potential of the RAPD type 9 strains in trophoblasts. To the best of our knowledge, this study is the first to use trophoblastic cell lines to assess the virulence potential of field isolates of *L. monocytogenes*. We found that the RAPD type 9 strains, as well as LO28, EGD and the maternofetal strain 3849-97 invaded the JAR cells at a lower level than the clinical strains (Figure 5.4) (Holch et al. 2009).

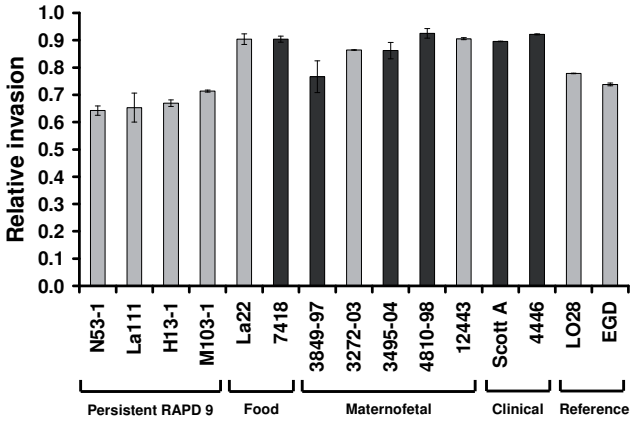

Figure 5.4: Invasion of *L. monocytogenes* strains into human trophoblastic JAR cells. Invasion is expressed as the number of intracellular CFU/ml relative to the number of CFU/ml added to the well. Strains have been sorted according to origin. Dark columns represent lineage I strains and light columns represent lineage II strains. Columns depict averages from one trial carried out in duplicate. Error bars indicate standard deviations. The results are representative of three independent experiments. From (Holch et al. 2009).

The lower invasion of the InlA-deficient RAPD type 9 strains and LO28 is consistent with the *in vitro* invasion of *inlA* mutants in both BeWo and JAR cells (Lecuit et al. 2004;Bakardjiev et al. 2004).

We also speculated that the ability to infect guinea pig fetuses could be reflected in an increased ability of the RAPD type 9 strains to multiply to higher numbers once inside the placental cells. However, we did not observe any differences in intracellular growth, indicating that the RAPD type 9 strains do not have any intracellular growth advantage compared to the clinical strains in this study (Holch et al. 2009).

Preceding growth conditions could probably affect the invasion into trophoblastic cells, as observed for Caco-2 cells (Garner et al. 2006b;Andersen et al. 2007;Werbrouck et al. 2009;Olesen et al. 2009). We speculated that the enhanced ability of the RAPD type 9 strain to invade guinea pig fetuses could be a result of a response to the stresses met during infection such as the antimicrobial compounds of the innate immune system. The RAPD type 9 strains did not exhibit an increased tolerance to the HDPs used in this study compared to other strains when analyzed *in vitro* in endpoint assays (Gottlieb et al. 2008). To test whether HDPs could induce a response in *L. monocytogenes* that would alter the infective capability, we pretreated *L. monocytogenes* strains with plectasin prior to infection of JAR cells (Figure

5.5). Also, this provided a more realistic model of infection, since *L. monocytogenes* is confronted with an array of HDPs during its passage through the gastrointestinal tract and dissemination in the blood. We compared the invasion of the RAPD type 9 strain La111 to a high invasive lineage I strain. Both strains have a MIC of 64 µg/ml for plectasin (Gottlieb et al. 2008). The invasion assay was done as described previously (Holch et al. 2009) with preparation of the plectasin-treated bacterial cultures as described in the legend of Figure 5.5.

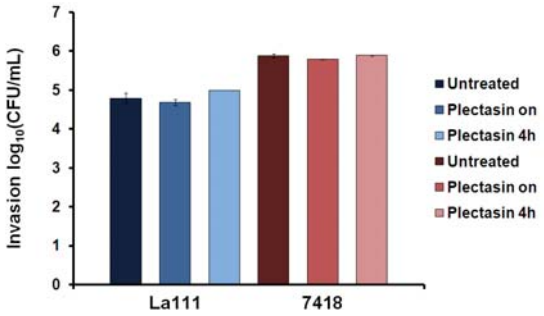

Figure 5.5: Invasion of JAR cells after exposure to plectasin. Bacterial cultures were prepared by inoculating fresh colonies (strains La111 and 7418) into MHB pH 7.4 broth. Untreated samples were incubated o.n. at 37°C and samples treated with plectasin for four hours were incubated at 37°C o.n. and plectasin added to a final concentration of 16 µg/mL (corresponding to ¼ MIC) 4 hours prior to infection of the cells. Samples treated with plectasin o.n. were added plectasin to a final concentration of 16 µg/mL and incubated o.n. at 37°C. At the day of the assay, bacteria were adjusted to 1×10^6 CFU/mL and used to infect the JAR cells as described earlier (Holch et al. 2009). Both strains were tested in duplicate (Gottlieb, unpublished data).

As observed earlier, the two strains differed in invasive ability, however, the pretreatment with plectasin did not affect the invasive ability. Since the preliminary data did not suggest that the response of RAPD type 9 to pre-treatment with the HDPs of the host innate immune defense was different from other strains, this experimental approach was not continued (Gottlieb, unpublished data).

5.2.2. Cell-to-cell spread

The intracellular cell-to-cell spread of *L. monocytogenes* is assessed by plaque forming assays, analyzing the ability of *L. monocytogenes* to form plaques of infected cells in a confluent cell monolayer. The advantage of plaque assays over the invasion assay described above is that it – in addition to the invasion and intracellular multiplication – also takes into account the extra parameter of cell-to-cell spread. Accordingly, a plaque forming assay in

Caco-2 cells showed higher specificity than an invasion and multiplication assay in discriminating between virulent and non-virulent *L. monocytogenes* strains as determined by intraperitoneal infection in immunocompromised mice (Van Langendonck et al. 1998). Also, plaque forming in HT-29 cells has been shown to be equivalent to subcutaneous infection of mice with respect to distinguishing virulent and non-virulent strains (Roche et al. 2001). It was furthermore found that strains belonging to serotype 4a, 4c and 4d that rarely cause listeriosis in humans formed smaller plaques compared to serotype 4ab and 4b strains, and this correlated with virulence determined by intraperitoneal infection of mice (Sokolovic et al. 1996).

Animal studies of pregnant guinea pigs and mice have shown that ActA-mediated cell-to-cell spread plays a major role in maternofetal infection in both guinea pigs and mice (Bakardjiev et al. 2005;Le Monnier et al. 2007). Consequently, we hypothesized that the RAPD type 9 strains would have an increased ability to spread from cell-to-cell. We analyzed the cell-to-cell spread in murine fibroblast cells, L929, as described earlier (Sun et al. 1990) mimicking the spread through the connective tissue of the placental villi to the fetal blood vessels. We found that the strains differed with respect to both the number and size of plaques (Figure 5.6).

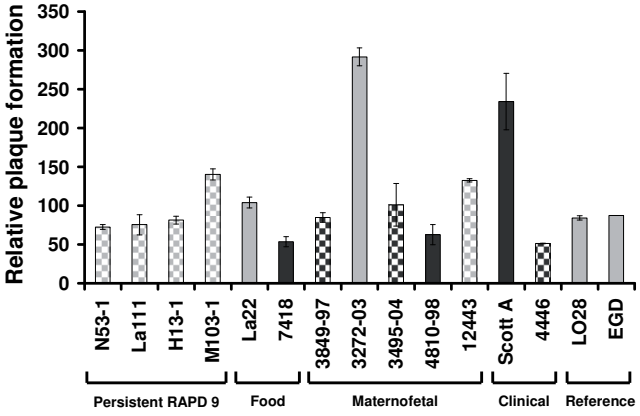

Figure 5.6: Plaque formation by L. monocytogenes in mouse fibroblastic L929 cells. Data is expressed as the number of plaques for 102 invaded bacteria. Strains have been sorted according to their origin. Dark columns represent lineage I strains and light columns represent lineage II strains. Check patterned columns represent strains with a 105 bp deletion in actA. Columns represent averages from one trial carried out in duplicate. Error bars indicate standard deviations. The results are representative of three independent experiments. From (Holch et al. 2009).

There were, however, no differences in either the number or size of plaques between strains when grouped according to origin. We did however observe that lineage I strains formed larger plaques than lineage II strains ($p < 0.05$) (Holch et al. 2009). Consistently, others have found that although no clear correlation between lineage and plaque size or plaquing ability (i.e. the number of CFU necessary to form one plaque) were found, lineage I strains tended to form more and larger plaques compared to lineage II strains (Wiedmann et al. 1997).

Another study showed that strains isolated from perinatal cases of listeriosis all showed similar virulence with regard to plaque formation in HT-29 cells as well as immune response measured as cytokine production from cord blood cells. Interestingly, the study tested strains from all three lineages and showed that also the strains from lineage II and III that are believed to be less virulent had the same virulence potential as the lineage I strains (Mereghetti et al. 2004). However, the results might be biased since all strains used in the study had already caused invasive disease. Hence, it is possible that other strains from the heterogeneous lineage II and III would have lower virulence potential.

5.3. Blood-brain barrier

Both the central nervous system (CNS) and the placenta are immunologically privileged compartments. Hence, the tissue tropism of *L. monocytogenes* for these tissues might be a common strategy of immune evasion, and lessons learned from the CNS infections due to *L. monocytogenes* might confer to placental infections as well.

The blood-brain barrier is constituted of the brain capillary endothelial cells separating the blood and the brain (Drevets et al. 2004). The concurrence of CNS infections and bacteremia suggest that blood-borne *L. monocytogenes* is the predominant route of CNS infections, as is the case for placental infections (Berche 1995;Drevets et al. 2004). Hence, direct invasion of endothelial cells lining the brain blood vessels is a route of entry. *L. monocytogenes* has been shown to enter human umbilical vein endothelial cells (HUVEC) (Drevets et al. 1995;Greiffenberg et al. 1997;Parida et al. 2002), and as *L. monocytogenes* also has to interact with fetal endothelial cells in the placental villi, this model is also relevant for crossing of the placental barrier (Parida et al. 2002). Different reports exist on the dependence of internalins in this process. While internalins were demonstrated to play a role in HUVEC-invasion (Drevets et al. 1995;Parida et al. 2002), Greiffenberg et al. found that entry into HUVEC occurred independently of InlA, InlB, InlC and ActA (Greiffenberg et al. 1997). However, in the latter study, HUVEC were grown in the presence of pooled human sera as opposed to fetal calf serum in the two first studies. It has later been shown that normal human serum contains *L. monocytogenes*-specific antibodies which inhibit the invasion into

human brain microvascular endothelial cells, HBMEC (Hertzig et al. 2003). Accordingly, an InlB-dependent entry of *L. monocytogenes* into HBMEC was observed (Greiffenberg et al. 1998).

Together with the apparent rather frequent asymptomatic carriage of *L. monocytogenes* by humans (Jacquet et al. 2004;Nightingale et al. 2005) the study by Hertzig et al. suggest that the extracellular bacteria in the blood stream as well as the endothelial cells are bathed in anti-listerial plasma proteins *in vivo* (Hertzig et al. 2003). This questions whether direct cell invasion of extracellular bloodborne *L. monocytogenes* into the barrier cells of the secondary target organs, the brain and placenta, actually plays a significant role *in vivo*.

5.3.1. Heterologous cell-to-cell assays

It has been suggested that *L. monocytogenes* (and other intracellular bacterial pathogens) could use infected mononuclear phagocytes as vectors to avoid host defenses and move from the bloodstream to immunologically privileged compartments. The concept of this Trojan horse model is that leukocytes are infected in the periphery and then transport intracellular *L. monocytogenes* to the CNS and across the blood-brain barrier. It has been shown that both *in vitro* and *in vivo* approximately 30% of the blood borne *L. monocytogenes* are associated with peripheral blood leukocytes (Drevets 1999;Drevets et al. 2001). Consequently, in addition to direct invasion, *L. monocytogenes* can also invade endothelial cells by heterologous cell-to-cell spread from infected macrophages both *in vitro* (Drevets et al. 1995;Greiffenberg et al. 1998) and *in vivo* (Drevets 1999;Drevets et al. 2001;Join-Lambert et al. 2005). Infected macrophages can also serve as vectors for heterologous spreading of *L. monocytogenes* into Caco-2 cells, COS-1 monkey kidney fibroblasts, and TIB-73 mouse hepatocytes (Greiffenberg et al. 1998). Specifically, Drevets et al. demonstrated that leukocyte-associated *L. monocytogenes* were fully capable of establishing CNS infection *in vivo* in the absence of extracellular bacteria due to continuous infusion of gentamicin (Drevets et al. 2001).

It is reasonable to assume that *L. monocytogenes* uses the same Trojan horse strategy to cross the placental barrier and hence, it would have been more realistic to investigate the heterologous cell to cell spread from infected phagocytes to trophoblasts rather than the direct invasion. However, preliminary attempts to set up an experimental model of heterologous cell invasion between *L. monocytogenes*-infected PMNs isolated from human whole blood and trophoblastic JAR cells did not succeed but provide basis for further research into this area (Gottlieb, unpublished results).

5.4. The virulence potential of RAPD type 9 strains – current status

The limitation of the cell models is that they assay single specific steps of the infectious process. In addition, they do not take into account the other factors such as the host immune defense that influence the infection *in vivo*. Hence a high virulence potential in one model does not necessarily mean that the strains have a high virulence *in vivo* as they can be attenuated in virulence in other steps of the infectious process. Indeed, in contrast to the many studies describing an association between high invasive ability and *in vivo* virulence, some studies have demonstrated that clinical strains have low invasive ability in Caco-2 cells and HepG2 cells (Werbrouck et al. 2006;Roberts et al. 2009). This emphasizes that clinical strains cannot a priori be regarded as high virulent in *in vitro* assays and also that other strain-specific characteristics such as the ability to survive and grow in the food matrix within which it is delivered to the host might affect the likelihood of these strains to cause disease (Roberts et al. 2009). In addition, the lower invasion capacity in cells that also correlated with a lower induction of pro-inflammatory cytokines might be a mechanism of immune invasion of clinical strains (Werbrouck et al. 2006).

We hypothesized that the ability of the otherwise low-virulent RAPD type 9 strains to infect guinea pig fetuses was a reflection of a specific ability to execute one or several of the infectious steps specific for maternofetal infection. We found that the RAPD type 9 strains did not have an increased ability to invade placental trophoblasts, nor did they exhibit increased intracellular growth rates. These results suggest that the RAPD type 9 strains do not have an increased ability to perform the first step of placental invasion, being the infection of trophoblasts. However, as discussed above, an indirect invasion through infected macrophages might be a more realistic experimental approach to assess the virulence potential in this step of the placental infectious cycle. Our subsequent experiments addressed whether the RAPD type 9 strains had an increased ability to spread from cell to cell, mimicking the spread through the connective tissue in the placental villi. The RAPD type 9 strains did not form more or larger plaques than the clinical strains used in this study, suggesting that they are not more efficient than other cells to reach the placental blood stream once inside the connective tissue of the villi.

The discrepancy between the low *in vitro* virulence potential of the RAPD type 9 strains and the *in vivo* infection of guinea pig fetuses is probably explained by the species specificity of the interaction of *L. monocytogenes* internalins with the host cell receptors. InlA interacts with human and guinea pig but not with mouse E-cadherin receptors (Lecuit et al. 1999), while InlB interacts with human and mouse but not with guinea pig Met receptors (Khelef et al.

2006) (Figure 5.7). Like humans, the gerbil is permissive to both the InlA – E-cadherin and the InlB – Met pathways.

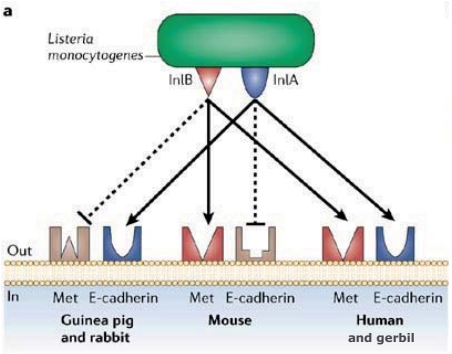

Figure 5.7: The species specificity of InlA – E-cadherin and the InlB – Met interaction. InlA recognizes human, gerbil and guinea pig E-cadherin but does not bind mouse E-cadherin due to a single amino acid substitution at position 16. InlB recognizes human, gerbil and mouse Met but do not recognize guinea pig Met for unknown reasons. From (Hamon et al. 2006).

During the course of this study, it has been demonstrated that InlA and InlB have interdependent roles in maternofetal infection in gerbils. Consequently, *L. monocytogenes* targets the placenta *in vivo* only if both the InlA and the InlB pathways are functional (Disson et al. 2008). Thus, the infection of guinea pig fetuses by the RAPD type 9 strain might be due to other pathways involved in infection, or other factors specific to either the host or the strain of *L. monocytogenes* that influence the course of infection.

In summary, we did not observe an increased virulence potential of the RAPD type 9 strains in cell models mimicking steps involved in crossing the placental barrier. Based on these results and in accordance with our previous observations (Jensen et al. 2008a) and the findings by Disson et al. (Disson et al. 2008), we conclude that the ability of the RAPD type 9 strains to infect guinea pig fetuses is a consequence of species specific differences in the host cell receptors involved in internalization of *L. monocytogenes*. Thus, the RAPD type 9 strains, although highly virulent to guinea pig fetuses, do not appear to pose a particular risk in human maternofetal listeriosis.

Of note, we and others have shown that strains belonging to another persistent RAPD type, RAPD type 15, have high virulence potential in Caco-2 cells (Jensen et al. 2007a), human whole blood (Gottlieb, unpublished results) and in non-pregnant guinea pigs (Roldgaard et al. 2008). Thus, the low virulence potential of the RAPD type 9 strains can not be extrapolated to other persistent sub types. Indeed, persistent *L. monocytogenes* have been the cause of

both sporadic cases of listeriosis as well as a multistate outbreak in the US (Olsen et al. 2005b;Orsi et al. 2008) emphasizing the need for further research into the physiology of such persistent subtypes.

5.5. Conclusions

Mammalian cell lines can be used to assay the ability of *L. monocytogenes* to execute specific steps of the infectious process. The advantage of the plaque forming assay over invasion assays is that it also takes into account the extra step of intra- and intercellular cell-to-cell spread. We investigated the discrepancy between previous *in vitro* and *in vivo* determinations of the virulence potential of the persistent RAPD type 9 strains. The RAPD type 9 strains did not exhibit increased virulence potential in cell models mimicking the single steps of the transmission across the placenta membrane and we concluded that they still can be regarded as low virulent with respect to human listeriosis.

6. Concluding remarks

The host defense peptides (HDPs) constitute a central part of the antimicrobial effector molecules of the innate immune system of all living organisms and have retained antimicrobial activity through millions of years. Recently they have been shown to have immunomodulatory functions as well. Together, this has prompted a massive interest in HDPs as a new generation of antimicrobials. On the other hand, the ability of pathogenic microorganisms to resist the antimicrobial effects of the innate immune system, such as the HDPs, is an essential part of the pathogenesis.

Knowledge about the response of bacteria to exposure to HDPs, the effect of environmental stresses or stimuli on tolerance to HDPs and the possible development of resistance is essential in order to evaluate the potential of HDPs as novel antimicrobials.

Given that pathogenic microorganisms are natural resistant to at least the HDPs that they are naturally exposed to during infection in the host, we hypothesized that natural variation in tolerance within a pathogenic species would exist and that this also reflected differences in virulence. We tested a broad collection of *L. monocytogenes* and *S. aureus* strains representing different origins, subtypes and virulence-related phenotypic behavior against four model peptides representing each of the three classes of HDPs. These were protamine, a linear peptide rich in proline and arginine, the fungal defensin plectasin, and the two linear, α-helical cathelicidins, novispirin G10 and its derivate novicidin. By measuring the ATP leakage from bacteria during treatment with these peptides, we determined that they have different mechanisms of action. We found that within each species, strains were equally sensitive to the four HDPs. This suggest that natural tolerance to HDPs does not exist in a population of human pathogenic bacteria and hence that the potential therapeutic use of HDPs is not hampered by naturally occurring resistant bacteria.

Since exposure to various stresses elicits a stress response in bacteria that confer resistance to other stressors as well, the environmental conditions to which a pathogen is exposed prior to contact with the host can influence the ability to cause infection. We examined if exposure to three food-related stress conditions (5% NaCl, pH 5.5 and 5°C) would alter the tolerance of *L. monocytogenes* to subsequent HDP treatments. We found that under the experimental conditions used in this study, these stress factors did not affect the tolerance to HDPs.

The development of resistance to HDPs is considered unlikely since they have retained a central role as antimicrobials in the innate immune defense of all living organisms for millions

of years. However, many conventional antibiotics are also originally natural compounds, and spontaneous resistant mutants occur readily. We examined if single mutations, induced by transposon mutagenesis, could produce mutants of *L. monocytogenes* and *S. aureus* with increased tolerance to plectasin. We identified a mutant in *S. aureus* with the transposon inserted into the response regulator, *hssR*, with a two- to four-fold increased tolerance compared to the wildtype but we did not find any resistant mutants in *L. monocytogenes*.

Collectively, these results do not speak against the use of HDPs as novel antimicrobials. However, since HDPs are “dirty drugs” that target many biological functions simultaneously, development of resistance would have to occur through cumulative changes involving several genes, which will require a long selection period. HDP resistance has been provoked in *E. coli* and *Pseudomonas* spp. through repeated subculture in increasing concentrations of HDPs. Such experiments and analyses of which genes are responsible for such resistant phenotypes could provide a basis for future studies on HDPs and development of resistance. Eventually, a better understanding of the molecular basis of resistance to HDPs might provide insights on how to design synthetic peptides to circumvent resistance problems.

The *L. monocytogenes* collection used in this study comprises a subgroup of food processing persistent strains (RAPD type 9) that previously have been shown to have low virulence potential in simple *in vitro* models but infected guinea pig fetuses just as efficiently as a high-virulent strain. We hypothesized that this discrepancy could be explained by an increased ability of these strains to execute one or several of the steps involved in transmission of *L. monocytogenes* across the placenta membrane and that sequence differences in central virulence genes could be the cause. We found that the RAPD type 9 strains had a lower invasive ability in placental trophoblasts and that this probably was caused by the presence of premature stop codons in their *inlA*. In addition they did not show enhanced ability to spread from cell-to-cell. Based on these results we concluded that the RAPD type 9 strains can still be regarded as low virulent with respect to human listeriosis.

It has been suggested that *L. monocytogenes* use infected phagocytes as vectors to avoid host defenses and move from the bloodstream to immunologically privileged compartments such as the CNS and the placenta. Hence other factors such as the host defense mechanisms and interaction with other cell types probably influence the course of infection *in vivo*. Further studies on virulence and in particular the ability to cross the blood-brain and the placenta barrier could include the establishment of cell models that closer mimics the *in vivo* conditions such as the heterologous cell-to-cell spread assay.

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Paper 1

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Antimicrobial peptides effectively kill a broad spectrum of *Listeria monocytogenes* and *Staphylococcus aureus* strains independently of origin, sub-type, or virulence factor expression.

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Antimicrobial peptides effectively kill a broad spectrum of *Listeria monocytogenes* and *Staphylococcus aureus* strains independently of origin, sub-type, or virulence factor expression

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Abstract

Background: Host defense peptides (HDPs), or antimicrobial peptides (AMPs), are important components of the innate immune system that bacterial pathogens must overcome to establish an infection and HDPs have been suggested as novel antimicrobial therapeutics in treatment of infectious diseases. Hence it is important to determine the natural variation in susceptibility to HDPs to ensure a successful use in clinical treatment regimes.

Results: Strains of two human bacterial pathogens, *Listeria monocytogenes* and *Staphylococcus aureus*, were selected to cover a wide range of origin, sub-type, and phenotypic behavior. Strains within each species were equally sensitive to HDPs and oxidative stress representing important components of the innate immune defense system. Four non-human peptides (protamine, plectasin, novicidin, and novispirin G10) were similar in activity profile (MIC value spectrum) to the human β-defensin 3 (HBD-3). All strains were inhibited by concentrations of hydrogen peroxide between 0.1% – 1.0%. Sub-selections of both species differed in expression of several virulence-related factors and in their ability to survive in human whole blood and kill the nematode virulence model *Caenorhabditis elegans*. For *L. monocytogenes*, proliferation in whole blood was paralleled by high invasion in Caco-2 cells and fast killing of *C. elegans*, however, no such pattern in phenotypic behavior was observed for *S. aureus* and none of the phenotypic differences were correlated to sensitivity to HDPs.

Conclusion: Strains of *L. monocytogenes* and *S. aureus* were within each species equally sensitive to a range of HDPs despite variations in subtype, origin, and phenotypic behavior. Our results suggest that therapeutic use of HDPs will not be hampered by occurrence of naturally tolerant strains of the two species investigated in the present study.

Background

Antimicrobial peptides (AMPs) are widespread as bacterial inactivator molecules in the innate immune systems of insects, fungi, plants, and mammals. The peptides are also known as host defense peptides (HDPs) as they have other, immuno-modulatory functions besides the direct antimicrobial actions. Three broad categories of HDPs have been identified: the linear peptides with helical structures (e.g. LL-37), the cysteine stabilized peptides with beta-sheet (e.g. the defensins), and a group of linear peptides rich in proline and arginine that primarily have been identified in non-mammalian species [1-3].

The HDPs target a broad spectrum of bacteria [3] and recently, these peptides have been suggested as novel antimicrobials for treating bacterial infections [4,5]. Whilst the peptides are regarded as universal antibacterial compounds, little is known about the sensitivity spectrum of different strains of pathogenic bacteria. Such understanding would be an essential part of evaluating the potential of HDPs in treatment.

Whilst some known pathogens possess intrinsic resistance mechanisms indicating a central role for HDP resistance in pathogenicity it is generally assumed that acquisition of resistance towards a given HDP is relatively improbable [6]. However, the spectrum of sensitivity, e.g. measured as MIC may vary in a selection of strains that may differ in genes known to be involved in resistance such as the *dlt* operon or *mprF* in *S. aureus* [7,8]. Also, HDPs and other components of the innate defense system may be viewed as stress factors against which bacteria have developed many counter protective mechanisms.

The ability of pathogenic bacteria to overcome these defense systems is essential to establish an infection. Strains of a particular pathogenic organism are not equally virulent [9-12] and may also differ in sensitivity to stresses encountered [9,13,14]. However, the resistance of different strains to the stresses imposed by the host defense systems might also differ and be indicative of differences in virulence.

The purpose of the present study was to determine the natural variation in sensitivity of strains of two pathogenic species to host defense peptides and hydrogen peroxide. In addition, if any differences were found, to determine if this could be reflected by variation in the strains' phenotypic behavior, including expression of virulence-related factors. Four model-peptides were chosen to represent each of the three different peptide categories: protamine is a linear arginine-rich peptide originally isolated from salmon spermatozoa [15], the fungal defensin plectasin [5], and two cathelicidins, novispirin G10 [16] and its derivate novicidin. We used a collection of the two Gram-positive organisms, *Listeria monocytogenes* and *Staphylococcus aureus*, and selected strains carefully to reflect different important niches of the bacteria. *L. monocytogenes* is a foodborne pathogen infecting via the gastrointestinal epithelia [17] and *S. aureus* is community- or hospital acquired and gains access to the tissues and blood stream whenever the skin or mucosal barrier is damaged [18]. To ensure that the strain collection reflected a broad variation in phenotypic behavior, we also determined the expression of several virulence factors and behavior of the bacteria in simple eukaryotic models. We found that the *L. monocytogenes* and *S. aureus* strains were within each species equally sensitive to single components of the innate immune defense system and this was not paralleled by their differences in phenotypic behavior.

Methods

Strains and culture conditions

Experiments were carried out with a collection of 25 *Listeria monocytogenes* strains (Table 1) and 16 *Staphylococcus aureus* strains (Table 2) representing different lineages and serotypes (*L. monocytogenes*), *spa* types (*S. aureus*), and origins (food processing environment, food products, and human clinical isolates). Six *L. monocytogenes* strains were mutants of the EGD strain and were mutated in genes known to be involved in stress tolerance. The *S. aureus* collection comprised two deletion mutants, strains Sa113/Δ*mprF* [8] and Sa113/Δ*dltA* [7], known to be more sensitive to host defense peptides. Two of the *S. aureus* strains could not immediately be assigned to an existing *spa* type and we are in the process of acquiring the additional information that is needed to assign a new *spa* type to them. The strains were obtained from The National Institute of Aquatic Resources and The National Food Institute, Technical University of Denmark, Faculty of Life Sciences, University of Copenhagen, Denmark, Statens Serum Institut, Denmark, University of Würzburg, Germany and Campden Food and Drink Association, United Kingdom. Stock cultures were stored at -80°C in 4% (w/V) glycerol, 0.5% (w/V) glucose, and 2.0% (w/V) skimmed milk powder. The bacteria were grown in Brain Heart Infusion (BHI) broth (Oxoid, CM0225), Tryptone Soy Broth (TSB) (Oxoid, CM129), and cation-adjusted Mueller-Hinton II Broth (MHB) (Becton Dickinson, 212322) adjusted to pH 7.4. To avoid unspecific binding of host defense peptides to plastic ware and agar, all MIC determinations were carried out using polypropylene plastic ware and radial diffusion assays were carried out in MHB supplemented with 1% agarose (Invitrogen, 15510-027) as gelling agent.

Host defense peptides and oxidative compounds

Protamine was purchased from Sigma (P4020-5G). Plectasin, novicidin, and novispirin G10 were supplied by Novozymes A/S. Recombinant HBD-3 was purchased

Table 1: Origin, serotype, lineage, and MIC values of the *Listeria monocytogenes* strains used in the present study

Strain	Origin	Sero	Lin	MIC[a]						Ref
				Pro	Ple	NoC	NoS	hBD	H_2O_2	
La22	CS[b] salmon	1/2a	2	16	128	4	64	16/16	0.23	[42]
V518a	Fish processing	4b	1	32	64	8	64	-	0.94	[42]
N53-1	Fish processing	1/2a	2	16	128	2	32	16/32	0.47	[43]
No40-1	Fish processing	1/2a	2	16	128	4	64	-	0.47	[43]
R479a	CS salmon	1/2a	2	16	128	4	64	-	0.47	[42]
O57	Gravad salmon	1/2a	2	16	128	4	128	-	0.47	[44]
H13-1	Fish processing	1/2a	2	16	128	4	64	-	0.94	[43]
La111	CS salmon	1/2a	2	8	64	4	32	8/16	0.94	[45]
M103-1	Fish processing	1/2a	2	32	128	4	64	-	0.94	[43]
EGD	Wildtype	1/2a	2	8	64	4	64	16/16	0.47	[c]
2375	EGD *perR* del	1/2a	2	32	64	4	32	-	0.94	[46]
2374	EGD *perR* ins	1/2a	2	32	128	4	32	-	0.94	[46]
2275	EGD *dps* del	1/2a	2	16	64	4	64	-	0.94	[47]
2317	EGD *prfA* del	1/2a	2	16	128	4	64	-	0.94	[c]
2315	EGD *sigB* del	1/2a	2	8	64	4	64	-	0.94	[48]
2307	EGD *resD* del	1/2a	2	8	128	2	32	-	0.12	[d]
LO28	Wildtype	1/2c	2	8	64	2	16	-	0.47	[17]
4666	Human clinical	1/2b	1	8	64	8	64	-	0.18	[49]
4459	Human clinical	1/2a	2	16	128	4	32	-	0.23	[49]
7418	Spread. sausage	1/2b	1	32	64	4	64	4/8	0.18	[49]
4446	Human clinical	4b	1	16	64	4	64	16/16	0.47	[49]
6895	Ham	1/2a	2	16	128	4	96	-	0.35	[49]
7291	Pasta w chicken	4b	1	32	64	8	128	-	0.47	[49]
4239	Human clinical	1/2a	2	32	64	4	64	-	0.23	[49]
Scott A	Human clinical	4b	1	16	64	4	32	8/8	N.D.	[e]

[a] MIC values are given in μg/ml for the five human defense peptides and in % (V/V) for H_2O_2.
[b] CS: Cold-smoked
[c] The strains were kindly provided by Werner Goebel, University of Würzburg
[d] The strain was kindly provided by Marianne Halberg Larsen, University of Copenhagen, Faculty of Life Sciences.
[e] The strain was kindly provided by Campden Food and Drink Association, UK.

Table 2: Origin, *spa* type, and MIC values of *Staphylococcus aureus* strains used in the present study

Strain	Origin	*spa*	MIC[a]						Ref
			Pro	Ple	NoC	NoS	hBD	H_2O_2	
8325-4	Wildtype	t211	16	32	8	128	32/32	0.47	[50]
Sa113	Wildtype	t211	16	32	6	128	-	0.18	[51]
Δ*mprF*	Sa113 *mprF* del	t211	8	4	1	8	-	0.12	[8]
Δ*dltA*	Sa113 *dltA* ins	t211	8	2	0.5	2	-	0.23	[7]
14943	Pork meat	t012	16	8	8	256	32/64	0.23	[b]
15033	Pork meat	t216	32	8	8	128	-	0.23	[b]
B31369	Human, clinical	t216	16	16	12	256	64/64	0.47	[b]
796	Pasta salad	t230	16	8	6	128	64/64	0.47	[b]
J15033	Human, clinical	t230	16	8	8	128	-	0.23	[b]
2148-jvi	Mastitis	t518	16	1	4	64	32/32	0.47	[b]
K3-B2	French cheese	t524	16	1	4	128	32/64	0.23	[b]
B29997	Human, clinical	t548	16	16	4	128	-	0.23	[b]
KES 439	Human, clinical	Uk[c]	32	2	4	128	-	0.23	[52]
KES 626	Human, clinical	t1269	16	1	4	64	32/64	0.18	[52]
KES 735	Human, clinical	Uk[c]	16	16	4	128	-	0.47	[52]
KES 855	Human, clinical	t339	16	16	4	64	-	0.23	[52]

[a] MIC values are given in μg/ml for the five human defense peptides and in % (V/V) for H_2O_2.
[b] The strains were kindly provided by Jørgen Leisner, University of Copenhagen, Faculty of Life Sciences.
[c] Uk: Unknown.

from PeproTech (300-52). The host defense peptides were dissolved in 0.01% acetic acid/0.1% bovine serum albumin (Sigma, A7906). Hydrogen peroxide was purchased from Bie & Berntsen (MER 1.07209.1000).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of host defense peptides in liquid medium

The strain collection was tested for sensitivity to protamine, plectasin, novicidin, and novispirin G10 by determining their minimal inhibitory concentration (MIC) using a microbroth dilution method [19]. Colonies from a BHI agar plate incubated overnight were suspended in MHB pH 7.4 to a turbidity of 0.11–0.12 at 546 nm (approx. 1.0×10^8 CFU/ml) and diluted in MHB to a concentration of 5.0×10^5 CFU/ml. 90 μl of bacterial suspension was incubated with 10 μl of peptide solution in polypropylene 96-well plates (Nunc, 442587) for 18–24 h at 37°C. The peptide solutions were made fresh on the day of assay and diluted two-fold. The range of concentrations assayed were 0.031–32 μg/ml for novicidin, 0.125–128 μg/ml for protamine and novispirin G10, and 0.25–256 μg/ml for plectasin. MIC was the lowest peptide concentration at which visual growth was inhibited. The minimum bactericidal concentration (MBC) values were determined by plating l0 μl samples from wells with no visible growth onto BHI agar plates. MBC was the lowest concentration of each peptide of which 99.9% reduction of the initial inoculum was observed.

Determination of Minimal Effective Concentration (MEC) of host defense peptides and H_2O_2 in radial diffusion assay

The MEC of HBD-3 was assayed on a sub-selection of strains using a radial diffusion assay [20] with some modifications. In brief, MHB/1% agarose was supplemented with glucose to an end-concentration of 0.1% (w/V) to enhance the growth of *L. monocytogenes* and improve the visualization of the inhibition zones. Bacterial suspensions were prepared as described for MIC determination in liquid medium, mixed with melted MHB pH 7.4/1% agarose/0.1% glucose medium at 42°C to 5.0×10^6 CFU/ml, and 10 ml gel was poured into 90 mm Petri dishes. Following solidification on a leveling table, 1 mm wells were punched with a Pasteur pipette. Two-fold dilutions of HBD-3 were prepared in 0.01% acetic acid/0.1% bovine serum albumin to a concentration range of 0.25–256 μg/ml and 2 μl was added to each well. The plates were incubated overnight at 37°C and MEC was determined as the lowest peptide concentration at which an inhibition zone was observed. Each strain was tested in two independent trials.

A similar assay was performed to determine the MEC of hydrogen peroxide. The bacteria were prepared and mixed with MHB/1% agarose as described above and 50 ml gel was poured into 140 mm Petri dishes. The gel was allowed to solidify and 3 mm wells were punched. Hydrogen peroxide was serially diluted from stock (30%) in Millipore water and 10 μl was transferred to each well. The plates were incubated overnight at 37°C and the MEC was read as described above.

Extracellular virulence factors of S. aureus

S. aureus strains were examined for production of several virulence factors to determine their variation in phenotypic behavior. It was verified that all strains were *S. aureus* by analyzing their production of protein A and clumping factor A using the BactiStaph identification kit (Oxoid, R21144) as described by the manufacturer. The hemolytic activity of *S. aureus* strains was determined in a microplate hemolysin assay [21,22]. All strains were grown overnight in BHI at 37°C with shaking and samples of the cultures were centrifuged at 10,000 × g for 10 min. The supernatant was treated with 10 mM dithiotreitol and 50 μl two-fold dilutions were made in BHI in a microtiter plate with U-formed wells. Bovine erythrocytes were washed in 0.9% saline with 0.1% gelatin and 0.0043% sodium azide and 100 μl of a 0.5% suspension was added to the supernatant. The plates were incubated at 37°C for 30–45 min and the hemolytic activity was scored as follows: +++, strong hemolysis; ++, moderate hemolysis; +, weak hemolysis; (+), questionable hemolysis; -, no hemolysis. The assay was carried out in duplicate. Staphylokinase activity was examined as described earlier [23]. Plates containing fibrinogen were prepared by dissolving human fibrinogen (Kordia, FIB 3) in double strength TSB to a final concentration of 0.1% (w/V). To this was added 3% (w/V) agar at 55°C and incubated at this temperature for 10 min. Fetal bovine serum (Invitrogen, 10106-151) was added at 0.1% (V/V). Plates without added serum served as controls for non-specific effects such as might be due to high levels of protease. Isolated colonies of each strain were then streaked onto the plates and incubated overnight at 37°C. A clear zone surrounding the bacterial growth indicated staphylokinase activity. The assay was repeated in two independent trials. Catalase activity was determined using the capillary tube catalase test as described earlier [24]. Briefly, 3% (V/V) H_2O_2 were drawn into capillary tubes (1 mm in diameter), a bacterial colony was touched with the H_2O_2 tube, and the amount of gas production was scored semiquantitatively after 10 seconds. +(+), ++, ++(+), and +++ represent a few bubbles, moderate number of bubbles, many bubbles, and gas forcing the 3% H_2O_2 upwards in the capillary tube, respectively. Ten isolated colonies were tested for each strain in two independent trials. Carotenoid production was assessed by smearing isolated colonies on white filter paper. Finally the strains were analyzed for production of enterotoxins A, B, C, and D, and toxic shock syndrome toxin (TSST-1) using reversed passive latex agglutination kits (Oxoid, TD0900 and TD0940) as described by the manufacturer.

S. aureus *induced killing of* C. elegans

For a sub-collection of *S. aureus* strains, the virulence was assessed in *C. elegans* as described [25]. 20 μl overnight culture of each strain was spread onto Nematode Growth Medium (NGM) plates and incubated at 37°C over night. For each strain, about 100 L4 hermaphrodites of the *pha-1* (*e2123ts*) mutant [26] were transferred from NGM plates seeded with *E. coli* OP50 to the plates seeded with staphylococci and incubated at 25°C. The plates were scored for live and dead worms every 24 hours and 50% mortality was taken as the time when 50% of the initial number of worms were dead. At least three independent trials were performed for each strain.

L. monocytogenes *invasion into Caco-2 cells and induced killing of* C. elegans

The data included are based on work presented in [27,28]. In brief, Caco-2 cells (ATCC HTB 37) for the invasion assay were grown to monolayers in 96-well tissue culture plates. Overnight cultures of *L. monocytogenes* were adjusted to approximately 1.5×10^7 CFU/ml and allowed to infect the Caco-2 cells for 1 hour at 37°C. Extracellular bacteria were killed by incubation with 50 μg/ml gentamicin for 1 hour at 37°C before the cells were lyzed using 0.1% Triton X-100. The number of intracellular bacteria was determined by plate count. The *C. elegans* assay was performed as described above for *S. aureus* except that the bacteria were spread onto Luria-Bertani (LB) plates.

Human whole blood killing assay

A sub-selection of strains was tested for sensitivity to human whole blood by incubating the individual *L. monocytogenes* and *S. aureus* strains with blood to a final concentration of 75%. Human blood samples were obtained from a normal healthy volunteer by venous puncture and collected in BD vacutainers coated with 3.8% citrate (Hettich Labinstruments Aps, 455382). Bacterial suspensions were prepared as described for MIC determination and diluted in MHB to a final concentration of approximately 5.0×10^3 CFU/ml, followed by addition of fresh human blood or peptone saline (0.1% peptone, 0.85% NaCl) as a control. *E. coli* MG1655 was used as a positive control for neutrophil-mediated killing. The mixtures were shaken (300 rpm) at 37°C for 24 hours. To determine bacterial viability, aliquots were withdrawn at the beginning of the assay and after 2, 4, 6, and 24 hours of incubation and serial dilutions were plated onto BHI agar. Each strain was tested in duplicate in two independent trials.

Statistical analysis

Data were analyzed using GraphPad Prism Statistical software. Data did not follow a Gaussian distribution and so Friedman's test was used to compare strains and Kruskal-Wallis test was used to compare groups. Dunn's post test was used for both. If only two groups were compared, the Mann-Whitney test was used. Since MIC and MEC values were determined from two-fold dilutions of peptides, these data were $\log_2$-transformed before test.

Results

MIC and MBC of HDP in liquid medium against L. monocytogenes *and* S. aureus

We compared the sensitivity of 25 *L. monocytogenes* strains and 16 *S. aureus* strains to four model HDPs (Table 1 and Table 2). The MIC values of the four HDPs against *L. monocytogenes* were 8–32 μg/ml (protamine), 32–128 μg/ml (plectasin), 2–8 μg/ml (novicidin), and 4–128 μg/ml (novispirin G10). For *S. aureus* the range was 8–32 μg/ml (protamine), 1–32 μg/ml (plectasin), 0.5–12 μg/ml (novicidin), and 2->128 μg/ml (novispirin G10). Protamine appeared to be equally efficient against both *L. monocytogenes* and *S. aureus*, while plectasin was more active against *S. aureus* than *L. monocytogenes*. Both novicidin and novispirin G10 were equally effective against the two bacteria and novicidin was clearly more potent than its parent peptide. The minimum bactericidal concentrations (MBC) of plectasin, novicidin, and novispirin G10 were identical to the MICs, suggesting that all three peptides have pronounced bactericidal effects.

The two peptide-sensitive *S. aureus* mutants (SA113/ΔmprF and SA113/ΔdltA) were, logically, more sensitive than the rest of the strains but otherwise there was no significant differences in peptide sensitivity between neither *L. monocytogenes* ($p = 0.0718$) nor *S. aureus* ($p = 0.0647$). Some differences were found between *L. monocytogenes* lineages in tolerance to single peptides (lineage 1 strains were more sensitive to plectasin, $p = 0.01$, and lineage 2 strains were more sensitive to novicidin, $p = 0.0334$), but there was no difference in tolerance between lineages to all the four peptides together ($p = 0.4627$). Likewise, no significant differences were found when the strains were grouped according to origin (clinical, food, and processing), suggesting that there is no systematic differences in peptide tolerance between the strains.

No systematic differences in MEC values of human β-defensin 3 between strains

A sub-selection of strains was tested against the human host defense peptide β-defensin 3 (HBD-3) to compare the activity spectrum to the four model-peptides. HBD-3 was originally identified in skin and is also expressed in epithelial cells lining the digestive tract and is active towards Gram-positive bacteria [29]. It is therefore highly relevant when examining both *L. monocytogenes* and *S. aureus*. The MEC values varied between 6 and 24 μg/ml for *L. monocytogenes* and 32 and 64 μg/ml for *S. aureus* (Tables 1 and 2). As for the non-human model-peptides, there was no systematic variation in MEC values when the

strains were grouped according to origin for neither *L. monocytogenes* (p = 0.2276) nor *S. aureus* (p = 0.2899). The MEC spectrum of HBD-3 was similar to the four non-human peptides.

MEC of hydrogen peroxide against L. monocytogenes *and* S. aureus *in radial diffusion assay*

The susceptibility of the strains to an oxidative burst generated by hydrogen peroxide was assayed by a radial diffusion assay (Tables 1 and 2). MEC values were in the range of 0.12–0.94% (V/V) against *L. monocytogenes* and 0.12–0.47% (V/V) against *S. aureus*. As was the case with the HDPs no systematic difference in tolerance between strains could be observed when grouped according to origin for neither *L. monocytogenes* (p = 0.0571) nor *S. aureus* (p = 0.5225). Also, there was no difference in tolerance between *L. monocytogenes* lineage 1 and 2 strains (p = 0.1491).

Determination of virulence factor expression and phenotypic behavior in L. monocytogenes *and* S. aureus *strains*

To address if differences in peptide sensitivity among strains reflect their virulence potential, it is necessary to have a collection of strains that represent a wide spectrum of virulence factor expression. We have previously shown that the *L. monocytogenes* strains used in the present study differ in behavior in several virulence factor assays. The strains varied in their ability to invade Caco-2 cells and in their killing kinetics against *C. elegans* [27,28]. A sub-collection of strains (Table 3) was selected for the present study to span the different behavior in the virulence models.

To select a similar representative sub-collection of *S. aureus* strains, each strain was analyzed for several extracellular virulence factors (Table 4).

The two HDP-sensitive mutants had significantly lower virulence factor expression than the rest (p = 0.0299), which could indicate that intrinsic HDP tolerance may be related to virulence of pathogenic bacteria. The rest of the strains varied in the expression of the individual virulence factors but no clear patterns in phenotypic behavior were found.

For instance, the clinical strains were not more hemolytic than the rest of the strains (p = 0.4676) consistent with reports on clinical strains without hemolytic activity [30,31]. In this study an animal clinical strain, 2148-jvi, was found to be non-hemolytic and two human clinical strains, KES 439 and KES 735, were only weakly hemolytic. Likewise, there was no correlation between the production of catalase and carotenoid (r = 0.444, p = 0.085), suggesting that these oxidative attack defense mechanisms contribute differently in each strain.

Three strains produced enterotoxins and only one of these, strain 14943, produced both enterotoxin A and TSST-1.

Seven strains were selected to represent different expression levels of the virulence factors and different strain origin. These were analyzed for their killing kinetics in a *C. elegans* worm model. The seven *S. aureus* strains killed the *C. elegans* more rapidly than the negative control strain *E.*

Table 3: Virulence assessment and survival in whole blood of a sub-collection of *L. monocytogenes* strains

Strain	Invasion Caco-2 (CFU/ml)	50% mortality *C. elegans* (hours)	Cell density Whole blood (CFU/ml)
La22	1.4×10^5	-	6.4×10^3
V518a	3.8×10^5	-	-
N53-1	1.9×10^2	110	1.3×10^3
No40-1	1.1×10^5	-	-
R479a	2.4×10^4	-	-
O57	4.1×10^4	-	-
H13-1	5.5×10^2	-	-
La111	4.1×10^2	110	2.9×10^2
M103-1	1.9×10^2	-	-
EGD	2.1×10^4	110	1.3×10^3
LO28	5.3×10^3	-	-
4666	3.1×10^5	-	-
4459	8.5×10^4	-	-
7418	2.8×10^5	80	2.8×10^4
4446	1.2×10^5	80	4.3×10^4
6895	3.3×10^4	-	-
7291	2.6×10^5	-	-
4239	1.9×10^4	-	-
Scott A	2.8×10^5	80	9.0×10^2

Data on Caco-2 cell invasion and *C. elegans* mortality based on [27,28].

Table 4: Virulence assessment of the *S. aureus* strain collection

Strain	Hemolysis	Staph. kin.	Catalase	Carotenoid	Enterotoxin				TSST-1	*C. elegans*	Blood
					A	B	C	D			
8325-4	+++	-[a]	++	+	-	-	-	-	-	184	2.4×10^8
Sa113	-	-	++	++	-	-	-	-	-		
Δ*mprF*	-	-	++(+)	++	-	-	-	-	-		
Δ*dltA*	-	-	+(+)	+	-	-	-	-	-		
14943	-	-	++(+)	+++	+++	-	-	-	+++	157	7.6×10^8
15033	++	-	+++	+++	-	+++	-	-	-		
B31369	++	-	++(+)	+++	-	+++	-	-	-	140	1.1×10^8
796	(+)	-	++(+)	+++	-	-	-	-	-	223	2.5×10^8
J15033	(+)	-	++	+++	-	-	-	-	-		
2148-jvi	-	-	++(+)	++	-	-	-	-	-	256	3.0×10^6
K3-B2	+	-	++	++	-	-	-	-	-	225	3.4×10^7
B29997	(+)	-	++(+)	+++	-	-	-	-	-		
KES439	(+)	-	++	++	-	-	-	-	-		
KES626	+++	-[a]	++(+)	+	-	-	-	-	-	118	1.0×10^9
KES735	(+)	-	++	+++	-	-	-	-	-		
KES855	++	-	++	++	-	-	-	-	-		

[a] A clearing zone on both plates with and without added serum indicate a high production of proteases.
Tests include hemolytic activity, staphylokinase activity, catalase activity, carotenoid production and production of exotoxins. A sub-selection of strains were tested for their killing kinetics against *C. elegans* (time to 50% mortality, h) and their ability to survive and grow in human whole blood (cell density after 24 h, CFU/ml).

coli OP50 (Figure 1 and Table 4). The strains differed with respect to the time taken to reach 50% mortality of the worms. When feeding on the two fastest killers, the human clinical strains KES 626 and B31369, the worms reached 50% mortality in 118 and 140 hours respectively. The three food isolates 14943, 796, and K3-B2 resulted in 50% mortality after 157 hours, 223 hours, and 225 hours respectively. Feeding on the laboratory reference strain 8325-4 took 184 hours to reach 50% mortality, while the mastitis isolate 2148-jvi led to 50% mortality in 256 hours.

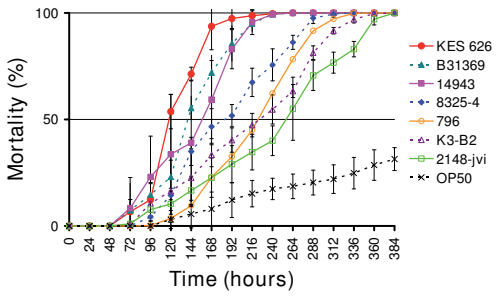

Figure 1
Assessment of virulence of a sub-selection of *S. aureus* strains against *C. elegans*. 100 *pha-1* mutant worms were tested for each strain. % mortality indicates the number of dead worms relative to the starting number of worms. Error bars represent standard deviations of triplicate measures.

Difference in ability to survive and grow in whole blood

A sub-collection of strains was examined for their behavior in human whole blood to analyze the response of these strains to a more complex model of the innate immune system. The strains were chosen on the basis of their origin and their behavior in the virulence factor assays described above (Table 3 and Table 4).

All seven *L. monocytogenes* strains were able to survive in human whole blood for 24 hours (Figure 2A). Strains EGD and Scott A remained at approximately the same cell number during the 24 hours of incubation, whereas the strains N53-1 and La111 grew during the first four hours of incubation and thereafter declined to the inoculation level or just below. The last three strains grew throughout the experiment. La22 increased approximately half a log unit and both 7418 and 4446 grew to approximately one log unit over inoculation level. The ability to grow and/or survive in whole blood was to some extent paralleled by the ability of the strains to invade Caco-2 cells and kill *C. elegans* worms. Thus, the better the survival or growth, the higher the invasion into Caco-2 cells and mortality in *C. elegans*. 7418 and 4446 both grew well in whole blood, were highly invasive in Caco-2 cells, and killed 50% of the *C. elegans* worms in 80 hours. Likewise, N53-1 and La111 whose cell numbers were declining in whole blood both were low invasive in Caco-2 cells and took 110 hours to kill 50% of the worms. For La22, EGD, and Scott A there

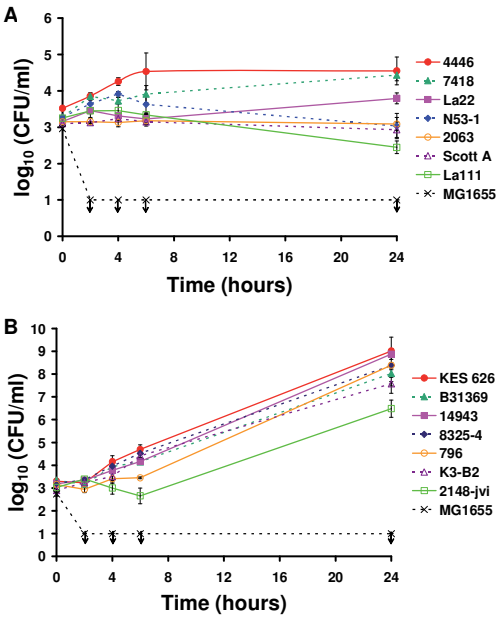

Figure 2
Survival of selected *L. monocytogenes* (A) and *S. aureus* (B) strains in human whole blood. Strains were adjusted to 1.0 × 10^3 CFU/ml, mixed 1:3 with human whole blood, and incubated at 37°C. *E. coli* MG1655 was used as a positive control for neutrophil killing. Bars represent standard deviations of duplicate observations. Graphs are representative of two independent experiments. Arrows indicate that cell numbers were below the detection limit (1.0 × 10^1 CFU/ml).

was not a clear pattern. There was a correlation between the invasive ability in Caco-2 and the lethality in *C. elegans* (r = -0.891, p = 0.033) but the survival in whole blood could not be correlated to neither Caco-2 invasion (r = 0.327, p = 0.498) nor *C. elegans* (r = -0.495, p = 0.356).

All seven *S. aureus* strains both survived and grew in whole blood (Figure 2B). Six of the strains had nearly identical growth patterns. KES 626 was the strain that reached the highest cell density (1.0 × 10^9 CFU/ml) after 24 hours. This was also the strain that killed the *C. elegans* worms the fastest. 2148-jvi grew remarkably poorer than the rest and reached a cell density of only 3.0 × 10^6 CFU/ml after 24 hours. When *C. elegans* fed on 2148-jvi, 50% mortality was not reached until after 256 hours compared to 118 hours for KES 626. There was however, no statistical correlation between killing time in *C. elegans* and survival in whole blood for the seven strains (r = -0.750, p = 0.066).

Together, the *C. elegans* and whole blood assays do not identify more virulent strains but they do indicate that the strains differ in phenotypic behavior.

Discussion

In the present study, we found that antimicrobial components of the innate immune defense (HDPs and hydrogen peroxide) act equally well on several strains of two pathogenic bacterial species (Table 1 and 2). We did not find significant inter-strain differences in sensitivity to neither the HDPs nor hydrogen peroxide. In general, the *S. aureus* strains were more sensitive to plectasin than *L. monocytogenes*, which is in concordance with previous findings [5]. The four non-human model peptides represent each of the three classes of host defense peptides and were similar in MIC spectrum to HBD-3.

Generally, *L. monocytogenes* strains from clinical cases are more virulent than strains isolated from environmental sources such as food [32]. Therefore, one could expect that the clinical isolates would be more tolerant to HDPs or hydrogen peroxide. However, no consistent patterns in tolerance were observed when the strains were grouped according to their origin.

The lack of difference between *L. monocytogenes* strains to the eukaryotic cationic peptides tested here is in contrast to the differences in sensitivity of strains to the bacterial cationic peptides, bacteriocins, that have been observed in several studies [33-35]. The differences could, however, not be correlated to neither strain origin [33] nor serovar [35] and may simply reflect the natural variation within the bacterial population.

There was no similarity in the *S. aureus* strains' performance in the virulence factor assays and their tolerance to the antimicrobial compounds of the innate immune defense system. Compared to *L. monocytogenes*, the disease spectrum of *S. aureus* is more complex. To assess the differences in phenotypic behavior of the *S. aureus* strains in parallel to the data we have obtained earlier for *L. monocytogenes* [27,28], we analyzed the *S. aureus* strains for several virulence factors that all contribute to evasion of the neutrophil attack at the site of infection [36]. There was considerably variation between the *S. aureus* strains for each of the single virulence factors tested in this study. However, none of the strains could be identified as generally more or less virulent than the rest and probably reflect that each strain is more or less specialized or virulent in one (or more) type(s) of infection(s).

The growth of *L. monocytogenes* and *S. aureus* strains in human whole blood and lethality in *C. elegans* demonstrated that the strains differed in growth in such eukaryotic systems but this could not be matched to tolerance to

host defense peptides or hydrogen peroxide. Likewise, there was no statistical correlation between the growth of *L. monocytogenes* in whole blood and the invasive ability in Caco-2 cells [27] or the lethality in *C. elegans* [28]. The ability of *S. aureus* to grow in human whole blood was not paralleled by any of the virulence parameters tested here. Liu and co-workers showed that disruption of the carotenoid biosynthesis impaired the resistance to both neutrophil and whole blood killing [37]. We found that strain 2148-jvi which had an intermediate carotenoid production grew poorly in human whole blood, whereas both 8325-4 and KES 626 which had a low carotenoid production grew very well in whole blood. There was no correlation between the *S. aureus* strains' growth in whole blood and their lethality in *C. elegans*, although the best and the poorest survivor (KES 626 and 2148-jvi, respectively) also were the fastest and the slowest to kill the worms. Blood from different donors may have different bactericidal activity and we initially compared the sensitivity of *L. monocytogenes* EGD to blood from three donors. The strain was equally sensitive to all three and the experiments were therefore performed with blood from only one donor.

The HDPs are widespread as a diverse and very well-conserved part of the defense system in all eukaryotes and have retained their antimicrobial activity for millions of years, hence the acquisition of resistance towards HDPs is considered unlikely. This has prompted the use of the design principles of these molecules for the design of new anti-infective drugs [3,38]. Indeed, several novel HDPs have been discovered and are thought to represent one of the most innovative families of anti-infective agents that have been characterized over the last 25 years [4,5]. In addition, HDPs have also been suggested as natural alternatives to chemical food preservatives [39-41].

Our data indicate that such host defense peptides would be well suited for both purposes as they appeared to have broad bactericidal effect on human pathogenic bacteria with different expression patterns of virulence factors. The lack of natural strains with a particular high tolerance to the peptides indicates that they are likely to be effective independently of the particular strain causing the infection.

Conclusion

We found that a collection of *L. monocytogenes* and *S. aureus* strains did not differ in tolerance to single components of the innate immune system despite representing a broad spectrum of phenotypically different organisms. Four non-human host defense peptides were similar in activity profile (MIC value spectrum) to the human HBD-3 when examined against a sub-selection of strains. When the same sub-selection was tested in human whole blood and *C. elegans* worms, differences between strains were found in each assay but there was no correlation between the two models. The broad activity of the HDPs against several strains of a pathogenic species indicates that natural resistance is not present in a population and these HDPs may indeed, as suggested, be useful as novel antimicrobials.

Authors' contributions

CTG carried out the MIC determinations, the testing of extracellular virulence factors in *S. aureus*, the human whole blood killing assays, and drafted the manuscript. LET carried out the *C. elegans* killing assay and helped to draft the manuscript. HI participated in the design of the study and helped to draft and revise the manuscript. PHM and HHK supplied the host defense peptides and participated in the design of the MIC determinations. LG participated in the design and coordination of the study and helped drafting and revising the manuscript. All authors read and approved the final manuscript.

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Paper 2

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The sensitivity of *Listeria monocytogenes* to host defense peptides is not influenced by innate or environmental stresses or by a key stress regulatory gene.

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The sensitivity of *Listeria monocytogenes* to host defense peptides is not influenced by environmental stresses or by a key stress regulatory gene

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ABSTRACT

Aims: Host defense peptides (HDPs) are explored as novel antimicrobials in the clinical setting and in foods. However, the sensitivity to HDPs may change if the bacterium is stressed before being exposed to HDPs. Our aim was to investigate the growth and survival of *Listeria monocytogenes* in the presence of HDPs and determine if growth under environmental stress conditions affected the tolerance to HDPs.

Methods and results. Growth and survival of *Listeria monocytogenes* EGD and *sigB* mutant in the presence of the HDPs plectasin or novicidin was followed by OD measurements and colony counts. Neither growth nor survival was affected in the *sigB* mutant. Three wildtype strains of *L. monocytogenes* were grown under food-related stress conditions (5% NaCl, pH 5.5 or 10℃/5℃) prior to exposure to HDPs. Pre-exposure to stress did not alter the strains' tolerance to subsequent HDP treatment.

Conclusions: SigmaB does not contribute to *L.* monocytogenes growth and survival of HDP treatment. In addition, pre-exposure of *L. monocytogenes* to a food relevant stress factor did not alter its sensitivity to HDPs.

Significance and impact of the study: Our results suggest that therapeutic use of HDPs will not be hampered by a stress response in pathogenic bacteria that would render them more tolerant to HDPs.

Keywords: *L. monocytogenes*, sigma B, host defense peptides, tolerance, environmental stress

INTRODUCTION

Host defense peptides (HDPs) are a central part of the innate immune defense of virtually every life form and have retained antimicrobial activity through millions of years, probably due to a delicate balance in the co-evolution of HDPs and microbes and their development of resistance mechanisms (Peschel & Sahl 2006). The broad antimicrobial activity and rapid action as well as their recently discovered additionally immunomodulatory properties, have prompted a massive interest in HDPs as a new generation of antimicrobials (Hancock & Sahl 2006). Also, the broader group of antimicrobial peptides (AMPs) are being researched as possible food preservatives (Potter, Hansen, & Gill 2005). However, the potential use of HDPs outside their natural environment e.g. in a clinical setting against “foreign” microbial pathogens might confer a different kind of selection pressure on the microbial pathogens, leading to development of resistance. In the context of HDPs being suggested as novel antimicrobials, it is important to understand the response of bacteria to these peptides and in particular if previous growth conditions (stress) would alter their sensitivity.

During the infectious process, pathogenic microorganisms encounter various stresses imposed by the innate immune system, including acidic and oxidizing conditions and the secretion of an arsenal of HDPs with different mechanisms of action. Hence, it could be speculated that the virulence potential is closely related to the ability of the pathogenic microorganism to cope with these stresses. Indeed, pathogenic bacteria with mutations rendering them less susceptible to HDPs have shown attenuated virulence (Abachin et al. 2002;Mandin et al. 2005;Peschel et al. 1999;Peschel et al. 2001;Thedieck et al. 2006), indicating the important role of tolerance to HDPs in pathogenicity and possibly also in virulence.

Listeria monocytogenes is a Gram-positive food borne pathogen that is capable of surviving and growing under conditions normally used in food conservation, including lowering pH, increasing the salt content and storage at cold temperatures (Farber & Peterkin 1991). Exposure to sub-lethal environmental stress conditions induce a stress response in *L.*

monocytogenes that renders it more tolerant to other forms of stress (Lou & Yousef 1997). The stress response is mediated by the Gram-positive central stress response regulator, SigmaB, that contributes to bacterial survival under adverse growth conditions, including high osmolarity, acid stress, cold stress and oxidizing conditions (Becker et al. 1998;Buncic et al. 2001;Ferreira, O'Byrne, & Boor 2001;Sue et al. 2004). SigmaB also regulates the expression of several virulence genes, including invasion-mediating *inlA* in *L. monocytogenes* (Garner et al. 2006a;Kazmierczak et al. 2003;Kim, Marquis, & Boor 2005) suggesting that SigmaB may contribute to the virulence of *L. monocytogenes*. The response of *L. monocytogenes* to bacterial antimicrobial peptides, bacteriocins, has also been shown to be dependent on SigmaB (Begley, Hill, & Ross 2006;Palmer, Wiedmann, & Boor 2009) and the other way around, antimicrobial peptides have been shown to activate the alternative sigma factor E in *Salmonella enterica* serovar Typhimurium and *Vibiro cholerae* (Crouch et al. 2005;Mathur, Davis, & Waldor 2007).

The purpose of this study was to investigate the growth and survival of *Listeria monocytogenes* in the presence of HDPs and determine if growth under environmental stress conditions prior to HDP treatment affected the tolerance of the bacteria to HDPs. We hypothesized that a *sigB* mutant was attenuated in growth and survival in the presence of HDP, and that exposure to food-related environmental stress factors could increase the tolerance of *L. monocytogenes* to HDPs. We found no effect of a deletion of *sigB* on the growth or survival of HDP treatments, neither could we demonstrate an increased tolerance to HDPs after stress adaptation to food-related environmental stress factors. Together, our results suggest that the potential use of HDPs will not be hampered by a stress response in pathogenic bacteria that would render them more tolerant to HDPs and thus lowering the therapeutic effect of HDPs.

MATERIALS AND METHODS

Strains and culture conditions

Experiments were carried out with a collection of six *Listeria monocytogenes* strains representing different origins (food processing environment, food products and human clinical strains), and different sub-types (RAPD-types, serotypes and lineages) (Table 1). Stock cultures were stored at -80°C in 4% (w/v) glycerol, 0.5% (w/v) glucose and 2.0% (v/w) skimmed milk powder. The bacteria were grown in Brain Heart Infusion (BHI) broth (Oxoid, CM0225) and enumeration was done on BHI agar (Oxoid, CM0225 and AppliChem, A7354). Treatments with HDPs were performed in cation-adjusted Mueller-Hinton II Broth (MHB) (Becton Dickinson, 212322) adjusted to pH 7.4. Plectasin and novicidin were supplied by Department of Antiinfective Discovery, Novozymes A/S. Peptides were diluted in peptide dilution buffer (0.01% acetic acid/0.1% bovine serum albumin) (Sigma, A7906).

Growth of *L. monocytogenes* EGD and *L. monocytogenes ΔsigB* in the presence of sublethal concentrations of HDPs

Overnight cultures of *L. monocytogenes* EGD and its *ΔsigB* mutant were inoculated (3%, 750 µL in 25 mL, 3:100) in MHB pH 7.4 and plectasin or novicidin was added to a final concentration of 16 µg/mL and 1 µg/mL, respectively, corresponding to ¼ MIC. The culture was incubated at 37°C with aeration (300 rpm) and OD_{546} was measured every two hours.

HDP-mediated killing of *L. monocytogenes* EGD and *L. monocytogenes ΔsigB*

To investigate if the stress response in *L. monocytogenes* was involved in responding to HDPs, *L. monocytogenes* EGD and *L. monocytogenes* EGD *ΔsigB* were treated with plectasin at MIC, two times MIC, and four times MIC. Colonies from a BHI agar plate incubated overnight were suspended in MHB pH 7.4 to a turbidity of 0.11-0.12 at 546 nm (approx. 1.0×10^8 CFU/ml) and diluted in MHB to a concentration of 5.0×10^5 CFU/mL. The bacterial suspensions were treated with plectasin, and incubated under aerated conditions (300 rpm) at 37°C. Cells treated with peptide dilution buffer were included as controls. To determine bacterial viability, aliquots were withdrawn at the beginning and after 1, 2, 4, 6, 8, and 24 hours of incubation and serial dilutions were plated onto BHI agar.

Treatment of *L. monocytogenes* with plectasin, novicidin, or a combination of both

Bacterial suspensions of 5×10^5 CFU/mL were prepared as described above and treated with plectasin, novicidin or a combination of both. Bacteria were treated with plectasin (64 µg/mL, corresponding to the MIC value) and with novicidin (1 µg/mL, corresponding to ¼ MIC), since the fast killing obtained when treating with MIC values of novicidin masked any effects of treating with the two peptides together. The cell suspensions were incubated at 37°C with aeration (300 rpm) and aliquots were withdrawn at 0, 1, 2, 4, 6, and 24 hours for determination of bacterial viability by plate count. Two independent experiments were performed.

Treatment with plectasin, novicidin, or a combination after pre-incubation under stressful conditions

Fresh colonies of *L. monocytogenes* N53-1, EGD, and 4446 from BHI agar plates were inoculated into TSB with 1% (w/V) glucose with or without stress. Stresses include 5% (w/V) sodium chloride, pH 5.5 (adjusted with 1 M HCl), and incubation at 10°C and 5°C. Bacteria treated with 5% NaCl or pH 5.5 were incubated 24 hours at 37°C with aeration (300 rpm), re-inoculated (100 µL in 5 mL) in media with or without the stress factor and incubated for another 24 hours at 37°C. Bacteria exposed to cold stress were inoculated in TSB with 1% glucose, and incubated for 4 days at 10°C, reinoculated in TSB 1% glucose and incubated 7 days at 5°C. For all experiments controls grown in TSB with 1% glucose at 37°C were included. After stress treatment, the bacteria were harvested by centrifugation (2,000 x g for 10 min), washed in MHB pH 7.4 and adjusted to 5×10^5 CFU/mL as described above. Plectasin was added to a final concentration of 128 µg/mL (N53-1 and EGD) or 256 µg/mL (4446) and novicidin was added to a final concentration of 4 µg/mL for all three strains. Samples were incubated at 37°C with aeration (300 rpm) and aliquots were withdrawn after 0, 2, 4, 6, 24, and 48 hours for enumeration by plate count on BHI agar. Each experiment was performed once.

RESULTS

No difference in growth between *L. monocytogenes* EGD and *L. monocytogenes* *ΔsigB* when treated with sublethal concentrations of HDPs

To investigate if SigmaB has a role in coping with the stress conferred by HDPs, we examined the growth of *L. monocytogenes* EGD and a *ΔsigB* mutant in the presence of sublethal concentrations of plectasin and novicidin. There was no difference between the wildtype and *ΔsigB* mutant in growth when treated with sublethal concentrations of plectasin and novicidin (Figure 1). In general, there was no difference in the growth of the EGD wildtype and the *ΔsigB* mutant, albeit the mutant grew slightly slower. Compared to when grown in peptide dilution buffer alone, cultures treated with plectasin had the same lag phase, but grew a little slower and went into stationary phase at a lower cell density. On the other hand, novicidin apparently act much more potent on the bacteria compared to plectasin, as even a ¼ MIC resulted in an 8 hour lag-phase.

Equal killing time for plectasin on *L. monocytogenes* EGD and *L. monocytogenes* *ΔsigB*

To investigate how SigmaB affected the survival of *L. monocytogenes* of treatment with HDPs, we examined the killing kinetics during plectasin exposure of L. *monocytogenes* EGD and a *ΔsigB* mutant (Figure 2). When treated with peptide dilution buffer alone, both strains grew equally well, indicating that the *sigB* deletion does not influence growth rate. When treated with plectasin at MIC, two times MIC and four times MIC values, there was no difference in killing time between the two strains, indicating that SigmaB does not contribute to survival in the presence of plectasin.

Treatment with plectasin at MIC, but not two and four times MIC, resulted in regrowth of both strains after eight hours. To determine whether regrowth was due to use or degradation of plectasin or an adaptation/ tolerance in the bacteria to plectasin, the MIC of plectasin was determined against the 24 h cultures treated with buffer and 64 µg/ml plectasin respectively. MIC was 64 µg/mL for the cultures treated with dilution buffer and 32 µg/mL for the cultures

treated with plectasin (data not shown). Thus, the cultures treated with plectasin have apparently not adapted to plectasin and it is not a plectasin-tolerant subpopulation that has emerged.

Additive effect of plectasin and novicidin against *L. monocytogenes*

So far, we have investigated the effect of treatment with single peptides, however, *in vivo* the pathogenic bacteria are met by an arsenal of different HDPs with different mechanisms of action that complement each other. Consequently, we also investigated the survival of *L. monocytogenes* in the presence of a combination of plectasin and novicidin to mimic the conditions at the mucosal surfaces and in the blood.

When treating bacterial suspensions with MIC concentrations of novicidin, the cell counts are immediately reduced by 2-3 log units (data not shown). Therefore, we determined which sub-inhibitory concentrations should be used in the combination treatment, so that the immediate effect of novicidin would not mask any combined effects of using the two peptides together and found that *L. monocytogenes* was mildly affected by 1 µg/mL (¼ MIC). Treatment with plectasin alone resulted in a 0.5 log unit reduction during the first eight hours of growth (Figure 3), and then the cells start to recover. Treatment with novicidin alone resulted in an immediate reduction of about one log unit, and the cell counts were further reduced by one log unit during the first hour of treatment before they started to recover. The combination of plectasin and novicidin resulted in a 3.5 log unit reduction in total during the first four hours of incubation before the cells started to recover. Thus, the effect of plectasin and novicidin seems to be additive (Figure 3). As mentioned above, the regrowth observed for all treatments is probably due to a degradation or use of the peptides.

Pre-incubation under stressful conditions do not alter the response of *L. monocytogenes* strains to HDPs

The use of the hurdle principles in food conservation can induce a stress response in food borne bacteria that renders them more tolerant to subsequent stresses e.g. of the immune

system (Andersen et al. 2007). To investigate if the stresses that *L. monocytogenes* is likely to meet during food conservation and storage would influence the tolerance to HDPs we pretreated three *L. monocytogenes* strains with three food preservation stresses: Growth at 5°C and 10°C, in 5% (w/V) sodium chloride, or at pH 5.5. Subsequently, *L. monocytogenes* strains were treated with plectasin, novicidin, or a combination of both.

There was no variation in the tolerance to the peptide treatments between the strains when they were grown in TSB 1% glucose without stress (data not shown), which is in accordance with our previous observations on the natural variability in peptide tolerance among strains of *L. monocytogenes* (Gottlieb et al. 2008). In addition, the pretreatment with 5% NaCl, pH 5.5, or 5°C did not affect the general growth ability of the strains as seen when the growth curves of the strains treated with peptide dilution buffer alone were compared (Figure 4d, 5d, and 6d). Pretreatment with 5% NaCl did not affect the tolerance to the subsequent treatment with plectasin (Figure 4a) or the combination of plectasin and novicidin (Figure 4c). There were some differences between the strains with respect to regrowth with novicidin treatment (Figure 4b), however the differences seem to be more strain-dependent than stress-dependent. No differences were observed between strains in tolerance to all three peptide treatments after pretreatment with low pH (Figure 5a-c) even though a diminutive regrowth was observed for N53-1 pretreated at pH 5.5, 4446 pretreated at pH 7.0, and EGD pretreated at pH 5.5. Incubation at 10°C and 5°C compared to 37°C did not alter the tolerance of the strains to plectasin (Figure 6a), except for EGD grown at 37°C that showed a remarkable lower tolerance than the rest of the strains. This result could however not be reproduced and is thus probably an artifact. As seen with pretreatment in the presence of NaCl, there were some differences in recovery after novicidin treatment (Figure 6b). Again, the difference appears to be more strain-dependent, although non-stressed cultures seem to recover better than stressed ones. The regrowth phenomenon is probably due to coincidence caused by small variations in inoculation levels or peptide concentrations between assays than it reflects actual strain- or stress differences.

Together, none of the three stressful conditions used in this study altered the tolerance of the strains to the subsequent peptide treatments, indicating that adaptation to food-related stresses does not increase the tolerance of the strains to HDPs. However, it is possible that any induced protection is abolished during harvesting from the stress media and suspension in a new media (without the stressor) for treatment with HDPs. It is noteworthy that the combination of plectasin and novicidin effectively killed all strains irrespective of origin and environmental stress conditions.

DISCUSSION

In this study we hypothesized that the HDPs of the innate immune system confer a stress upon the invading bacteria, and consequently that a mutant in the central stress response regulator, *sigB*, could be expected to be more sensitive to the stresses imposed by the host defense system. We have investigated the influence of HDPs on the growth and survival of *L. monocytogenes* EGD and its *ΔsigB* mutant. We have also examined whether pretreatment with food-relevant stress factors altered the tolerance to subsequent treatment with HDPs.

We found that there was no difference in the growth of *L. monocytogenes* EGD and its *ΔsigB* mutant in the presence of sublethal concentrations of the two HDPs plectasin and novicidin (Figure 1). Likewise, there was no difference in the survival of the wildtype and *ΔsigB* mutant of plectasin treatment (Figure 2). We have previously shown that *L. monocytogenes* EGD and the *ΔsigB* mutant had identical tolerances (MIC values) to four different HDPs representing each of the three classes of HDPs (Gottlieb, Thomsen, Ingmer, Mygind, Kristensen, & Gram 2008). Similarly, Begley and co-workers found that *L. monocytogenes* 10403S and a *sigB* deletion mutant had similar sensitivity to the bacteriocins nisin and lacticin when assessed in endpoint MIC assays (Begley, Hill, & Ross 2006). In contrast, both the growth and survival of treatments with the bacteriocins (as well as conventional,

membrane-active antibiotics ampicillin and penicillin G) were greatly attenuated in the *sigB* mutant (Begley, Hill, & Ross 2006). Others have found that the Gram-negative alternative sigma factor, SigmaE, is involved in the resistance of both *Salmonella enterica* serovar Typhimurium and *Vibrio cholerae* to the human HDP-derivate P2 (Crouch, Becker, Bang, Tanabe, Ouellette, & Fang 2005;Mathur, Davis, & Waldor 2007). The discrepancy to our results might be explained by different mechanisms of actions of the antimicrobials used in the studies. P2 targets the cell envelope of Gram-negatives (Weiss, Beckerditequagliata, & Elsbach 1980) and both the bacteriocins and conventional antibiotics are membrane active as well (Hechard & Sahl 2002). In contrast, plectasin does not appear to have classical membrane actions (Gottlieb et al. 2007;Mygind et al. 2005) and novicidin appears to at least have additional intracellular targets besides the bacterial membrane (Gottlieb, Mygind, Kristensen, & Gram 2007).

The regrowth observed both after treatment with both plectasin and novicidin (Figure 2 and 3) and in some cases also after treatment of stress-pretreated cultures (Figure 4, 5, and 6) is not caused by the emergence of resistant mutants, as the regrowth population does not show elevated MIC values to plectasin. It can instead be due to the presence of persister cells in the bacterial population. Persister cells are non-dividing, dormant cells that are tolerant to antimicrobial treatments since the target molecules for HDPs are not active and hence their function is not compromised by the antimicrobial (Lewis 2007). These will revive and regrow when the antimicrobial is gone, either because it is used (bound irreversibly to bacterial targets) or degraded e.g. by bacterial proteases.

The combination of plectasin and novicidin showed an additive effect on killing of *L. monocytogenes*. We have previously implied that these two peptides have different mechanism of action based on their different ATP leakage profiles (Gottlieb, Mygind, Kristensen, & Gram 2007;Thomsen et al. 2009). Synergistic effects of two antimicrobial peptides with different mode of actions, nisin and the polypeptide ε–poly-L-lysine, have been

observed for *L. monocytogenes* and *Bacillus subtilis* but not for *E. coli* (Najjar, Kashtanov, & Chikindas 2007). Bacteriocins are not active against Gram-negatives due to their outer membrane. Interestingly, combination of several bacteriocins with the fish AMP pleurocidin resulted in synergistic effects against *E. coli*, probably because pleurocidin renders the outer membrane permeable to the bacteriocins that then gain access to the inner membrane to exert their antimicrobial activity (Luders et al. 2003). The simultaneous use of several antimicrobials with different mode of actions appears also to be a general principle of innate immune defense (Peschel & Sahl 2006). In a therapeutic perspective, synergistic effects between HDPs would allow for administration of lower concentrations of antimicrobials that in turn could lead to a less favorable condition for the occurrence of HDP-resistant subpopulations (Bell & Gouyon 2003).

Since exposure of *L. monocytogenes* to environmental stress conditions have been shown to induce the expression of both stress response and virulence genes (Kazmierczak, Mithoe, Boor, & Wiedmann 2003;Olesen, Vogensen, & Jespersen 2009) and increase the phenotypic virulence potential (Garner et al. 2006b;O'Driscoll, Gahan, & Hill 1996;Olesen, Vogensen, & Jespersen 2009), we hypothesized that exposure of *L. monocytogenes* to food-relevant environmental stress conditions would initiate a stress response that would affect their tolerance to HDPs. However, we found that pretreatment of *L. monocytogenes* with food-related environmental stress factors did not affect the tolerance of *L. monocytogenes* to the HDPs used in this study (Figure 4-6). In contrast, acid adaptation at pH 5.5 (pH adjusted with lactic acid) has been shown to increase the tolerance to nisin and to a lesser extent lacticin. The acid adaptation altered the composition of fatty acids in the bacterial membrane and the differences in tolerance probably reflect the different mode of actions of the two bacteriocins (van Schaik, Gahan, & Hill 1999). Also, short term exposure (60 min) to either 6.5% NaCl or 5℃ have also been shown to protect *L. monocytogenes* to subsequent pediocin treatment (Jydegaard, Gravesen, & Knochel 2000).

Others have found that incubation under stressful conditions similar to those used in this study (7% NaCl, pH 4.5 – 5.0, and 4°C) increased the tolerance of *L. monocytogenes* to the oxidative effects of H_2O_2, another antimicrobial compound of the innate immune defense (Lou & Yousef 1997;Stecha et al. 1989). Growth at 4°C for 7 days actually increased the *L. monocytogenes* survival rate in human neutrophils (Stecha, Heynen, Roll, Brown, & Czuprynski 1989). In addition, acid adaptation has also been shown to increase the intracellular survival of *L. monocytogenes* in macrophages, probably due to increased expression of genes involved in response to acid and oxidative stresses (Conte et al. 2002). These studies all imply that stress adaptation to food-related stresses can render *L. monocytogenes* more tolerant to the stresses of the innate immune system and hence increase the virulence potential.

In this study, *L. monocytogenes* was pre-incubated under stressful conditions, harvested and then treated with HDPs under non-stressful conditions (normal osmolarity, pH 7.4, 37°C), thus the stress response could be lost during harvesting. However, treatment with HDPs under stressful conditions could hamper the effect of the HDPs and hence give false positive results for stress hardening with respect to HDP tolerance. It is well known that the presence of ions affect electrostatic attraction between HDPs and bacterial membrane (Brogden 2005;Zasloff 2002). In addition, both pH and temperature have been shown to affect protamine activity, probably due to alterations in the fatty acid composition of the cell membrane (Johansen et al. 1997), as also shown for bacteriocins (van Schaik, Gahan, & Hill 1999). Also, we subjected *L. monocytogenes* to stresses on a long term basis (adaptation) which has been shown to result in a different stress response when examining the transcriptional profile compared to the response to stress on a short term basis (shock) (Olesen, Vogensen, & Jespersen 2009). Some of the above mentioned studies observed the effects after short term stresses and it is possible that a short term stress induction could have resulted in altered HDP tolerance. However, it is important to study the response under

conditions relevant to the system where the pathogen is found, in this case in food, where the stresses are indeed implied on a long-term basis.

In conclusion, the data produced in this study does not speak against the use of HDPs as alternative antimicrobial therapeutics. Others have suggested concern that the use of HDP and the potential evolution of resistant bacterial populations would hamper our intrinsic defenses against infectious diseases (Bell & Gouyon 2003). Previously, we have observed that natural variation in tolerance to HDPs does not exist in a collection of *L. monocytogenes* representing different subtypes and virulence phenotypes (Gottlieb, Thomsen, Ingmer, Mygind, Kristensen, & Gram 2008). Here, we report that pre-exposure to food-related stresses do not alter the tolerance to subsequent HDP treatment as have been observed for several bacteriocins.

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TABLES

Table 1: Origin, serotype, and lineage of the *Listeria monocytogenes* strains used in the present study.

Strain	Origin	Serotype	Lineage	Ref
N53-1	Fish processing plant persistent	1/2a	2	(Wulff et al. 2006)
EGD	Wildtype	1/2a	2	*
2315	EGD *sigB* del mutant	1/2a	2	(Brondsted et al. 2003)
4446	Human, clinical	4b	1	(Larsen et al. 2002)

* The strain was kindly provided by Werner Goebel, University of Würzburg.

FIGURES

Figure 1: Growth of *L. monocytogenes* EGD wildtype and a *sigB* deletion mutant in the presence of sub-lethal concentrations of plectasin and novicidin.

Overnight cultures were diluted 3% in MHB pH 7.4 and treated with ¼ MIC (16 µg/mL plectasin and 1 µg/mL novicidin, respectively) at 37°C under aerated conditions (300 rpm). Treatment with plectasin does not have noticeable effects on the growth compared to buffer, whereas treatment with novicidin almost abolishes growth. Strains and HDP treatment: (■) EGD + Buffer, (□) Δ*sigB* + Buffer, (▲) EGD + Plectasin, (△) Δ*sigB* + Plectasin, (●) EGD + Novicidin, and (○) Δ*sigB* + Novicidin.

Figure 2: Plectasin-induced killing of *L. monocytogenes* EGD wildtype and a *sigB* deletion mutant.

The bacteria were adjusted to 5.0×10^5 cfu/ml and treated with plectasin at 256 µg/mL, 128 µg/mL, or 64 µg/mL or with peptide dilution buffer at 37°C. There are no differences in survival between EGD and the *sigB* deletion mutant. 256 µg/mL and 128 µg/mL plectasin killed both strains within 6-8 hours. Strains and HDP treatment: (■) EGD + 256 µg/ml plectasin, (□) Δ*sigB* + 256 µg/ml plectasin, (▲) EGD + 128 µg/ml plectasin, (△) Δ*sigB* + 128 µg/ml plectasin, (●) EGD + 64 µg/ml plectasin, (○) Δ*sigB* + 64 µg/ml plectasin, (♦) EGD + Buffer, and (◊) Δ*sigB* + Buffer.

Figure 3: Survival of *L. monocytogenes* after treatment with plectasin, novicidin, or a combination of both.

The bacteria were adjusted to 5.0×10^5 cfu/ml and treated with plectasin (64 µg/mL), novicidin (1 µg/mL), or a combination of plectasin and novicidin at 37°C. Bacteria treated with peptide dilution buffer alone were included as controls. There is an additive effect of plectasin

and novicidin in treatment of *L. monocytogenes*. HDP treatment: (■) Plectasin, (▲) Novicidin, (●) Plectasin + Novicidin, and (◊) Buffer.

Figure 4: Treatment of *L. monocytogenes* strains with plectasin, novicidin or a combination after pretreatment with or without 5% NaCl.

Strains were grown in TSB 1% glucose with (closed symbols, straight lines) or without (open symbols, dotted lines) 5% NaCl in two successive inoculations, harvested, washed, and resuspended in MHB pH 7.4 to app. 5×10^5 CFU/mL prior to treatment with (a) plectasin, (b) novicidin, (c) a combination of plectasin and novicidin, or (d) peptide dilution buffer. Strains and stress conditions: (■) EGD + NaCl, (□) EGD – NaCl, (▲) 4446 + NaCl, (△) 4446 – NaCl, (●) N53-1 + NaCl, and (O) N53-1 – NaCl.

Figure 5: Treatment of *L. monocytogenes* strains with plectasin, novicidin or a combination after pretreatment with pH 5.5 or pH 7.0.

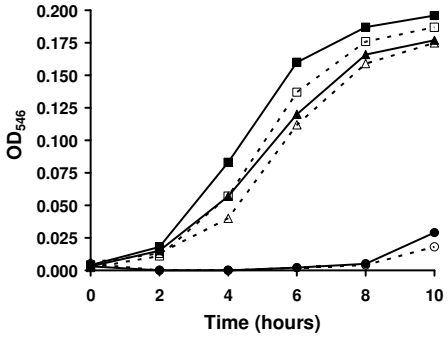
Strains were grown in TSB 1% glucose pH 5.5 (closed symbols, straight lines) or pH 7.0 (open symbols, dotted lines) in two successive inoculations, harvested, washed, and resuspended in MHB pH 7.4 to app. 5×10^5 CFU/mL prior to treatment with (a) plectasin, (b) novicidin, (c) a combination of plectasin and novicidin, or (d) peptide dilution buffer. Strains and stress conditions: (■) EGD pH 5.5, (□) EGD pH 7.0, (▲) 4446 pH 5.5, (△) 4446 pH 7.0, (●) N53-1 pH 5.5, and (O) N53-1 pH 7.0.

Figure 6: Treatment of *L. monocytogenes* strains with plectasin, novicidin or a combination after pretreatment at 10℃ and 5℃ or 37℃.

Strains were grown in TSB 1% glucose for four days at 10 ℃, reinoculated and grown at 5 ℃ for 7 days (closed symbols, straight lines) or grown at 37℃ o.n. in two successive

inoculations (open symbols, dotted lines). They were harvested, washed, and resuspended in MHB pH 7.4 to app. 5×10^5 CFU/mL prior to treatment with (a) plectasin, (b) novicidin, (c) a combination of plectasin and novicidin, or (d) peptide dilution buffer. Strains and stress conditions: (■) EGD 10℃/5℃, (□) EGD 37℃, (▲) 4446 10℃/5℃, (△) 4446 37℃, (●) N53-1 10℃/5℃, and (○) N53-1 37℃.

Figure 1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Time (hours)

Figure 2

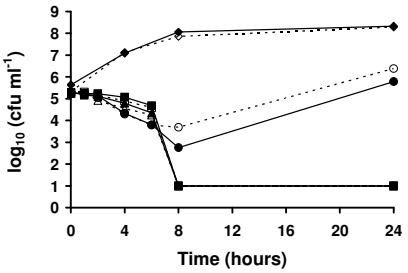


Figure 3

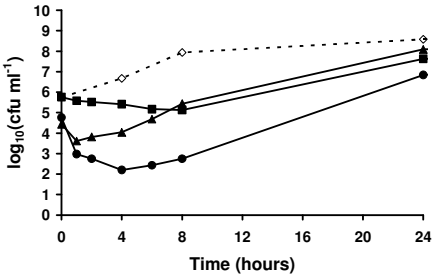


Figure 4

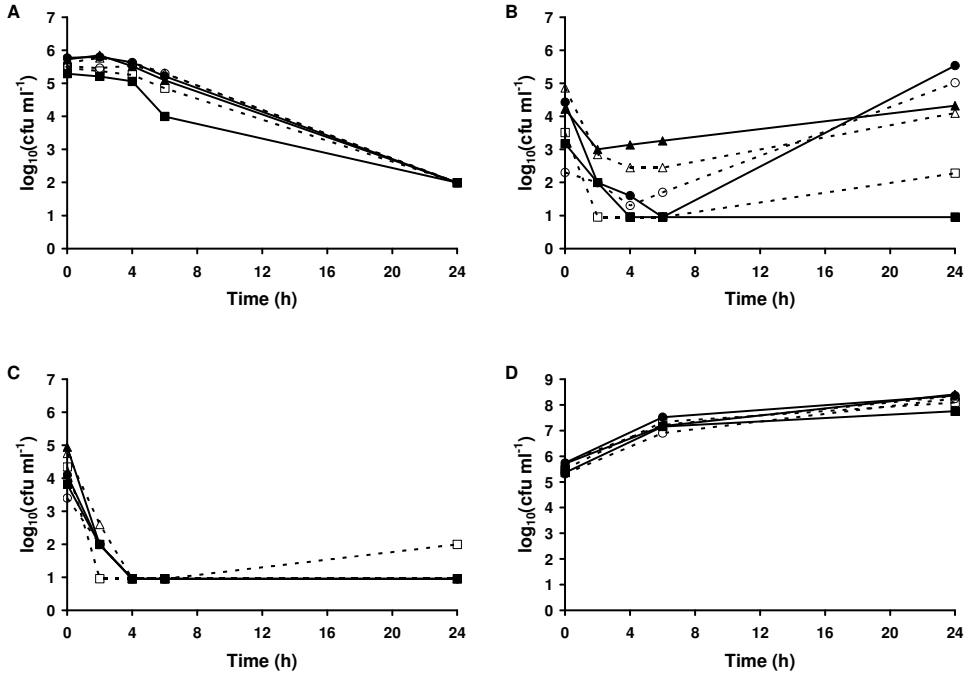


Figure 5

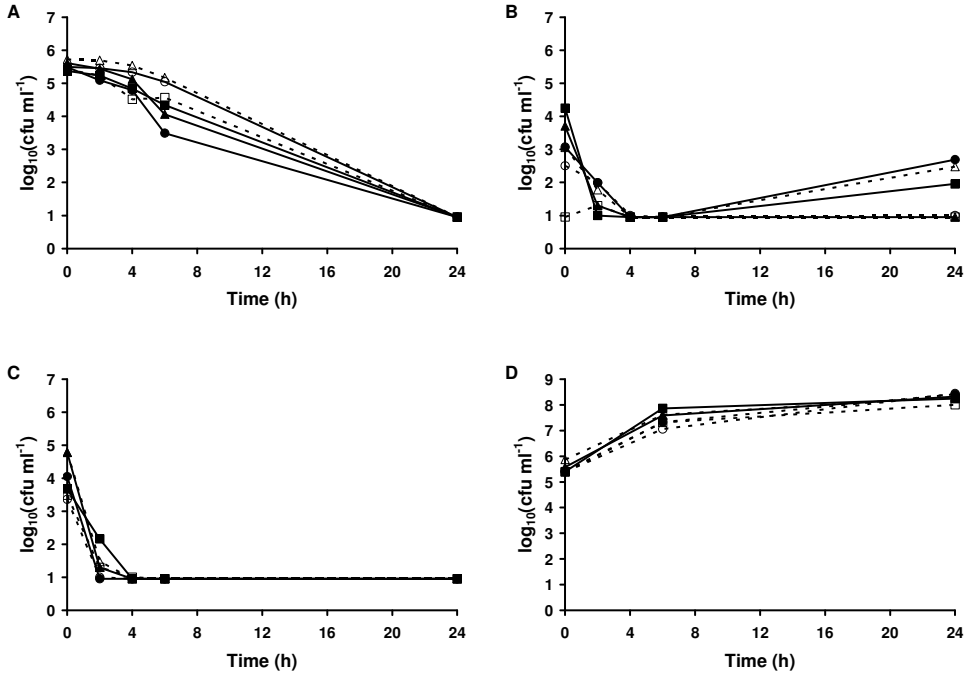
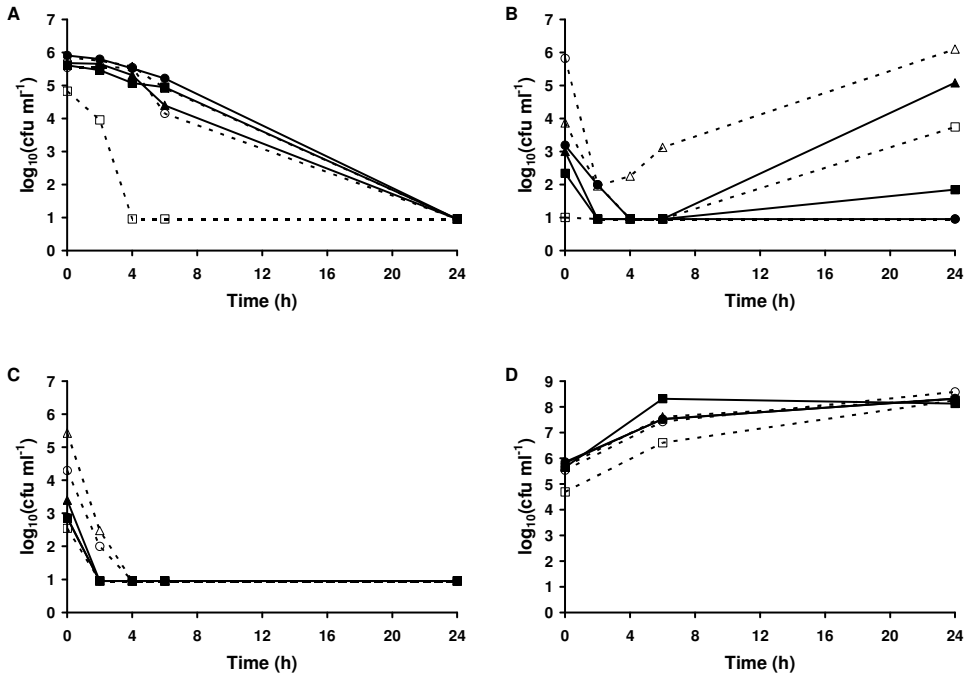


Figure 6



Paper 3

Line Elnif Thomsen, Caroline Trebbien Gottlieb, Sanne Gottschalk, Tim Tue Wodskou, Hans-Henrik Kristensen, Lone Gram, Hanne Ingmer (2009).

The heme sensing response regulator HssR in *Staphylococcus aureus* but not the homologous RR23 in *Listeria monocytogenes* modulates susceptibility to the antimicrobial peptide plectasin.

BMC Microbiology. Ready for submission, pending approval by Sanofi-Aventis.

The heme sensing response regulator HssR in *Staphylococcus aureus* but not the homologous RR23 in *Listeria monocytogenes* modulates susceptibility to the antimicrobial peptide plectasin

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Abstract

Background: Host defence peptides (HDPs), also known as antimicrobial peptides (AMPs), have emerged as potential new therapeutics and their antimicrobial spectrum covers a wide range of target organisms. However, the mode of action and the genetics behind the bacterial response to HDPs is poorly understood and such knowledge is required to evaluate their potential as antimicrobial therapeutics. Plectasin is a recently discovered HDP active against Gram-positive bacteria with the human pathogen, *Staphylococcus aureus* being highly susceptible and the food borne pathogen, *Listeria monocytogenes* being less sensitive. In the present study we aimed to use transposon mutagenesis to determine the genetic basis for *S. aureus* and *L. monocytogenes* susceptibility to plectasin.

Results: In order to identify genes that provide susceptibility to plectasin we constructed bacterial transposon mutant libraries of *S. aureus* NCTC8325-4 and *L. monocytogenes* 4446 and screened for increased resistance to the peptide. No resistant mutants arose when *L. monocytogenes* was screened on plates containing 5 and 10 fold MIC of plectasin. However, in *S. aureus*, two mutants with insertion in the heme response regulator (*hssR*) were 2- 4 fold more tolerant to plectasin as compared to the wild type. The *hssR* mutation also enhanced tolerance to the plectasin-like defensin eurocin, but not to other classes of HDPs or to other stressors tested. Addition of plectasin did not influence the expression of *hssR* or *hrtA*, a gene regulated by HssR. The genome of *L. monocytogenes* LO28 encodes a putative HssR homologue, RR23 (in *L. monocytogenes* EGD-e lmo2583) with 48% identity to the *S. aureus* HssR, but a mutation in the *rr23* gene did not change the susceptibility of *L. monocytogenes* to plectasin.

Conclusion: *S. aureus* HssR, but not the homologous RR23 from *L. monocytogenes*, provide susceptibility to the defensins plectasin and eurocin. Our data suggest that a functional difference between response regulators HssR and RR23 is responsible for the difference in plectasin susceptibility observed between *S. aureus* and *L. monocytogenes*.

Background

Humans are living in a constant struggle with infectious microorganisms and whilst improved hygiene has been essential to control such organisms, one of the major steps forward has been the discovery and use of antibiotics. However, the high rate at which bacteria become resistant to currently used antibiotics is regarded as a major threat to the future treatment of infectious diseases in both humans and livestock [1,2] Therefore, there is a growing demand for new types of antimicrobial compounds and interest is focused on host defence peptides (HDPs) as novel therapeutic agents. HDPs are a unique and diverse group of peptides, which can be grouped into different classes, based on their amino acid composition and structure. In humans and other mammals, the defensins and the cathelicidins constitute the two main HDP families. The cathelicidins vary widely in sequence, composition and structure [3], but share a highly conserved N-terminal structural domain (cathelin) linked to a highly variable cathelicidin peptide domain. The defensins are more uniform, small cystein-rich cationic peptides [4]. Defensins have well-established antimicrobial activity against a broad spectrum of pathogens, and in addition they have been shown to have immunostimulatory functions on both innate and adaptive immunity [5]. This has prompted a massive interest in synthetic defensins as novel antimicrobial candidates for therapeutic use.

Recently, the antimicrobial peptide, plectasin isolated from a saprophytic fungus, was described [6]. Plectasin is a defensin, which has broad activity against several species of Gram-positive bacteria [6,7] and combined with very low toxicity in mice and on human keratinocytes and erythrocytes, plectasin holds promises as a novel antiinfective treatment [6].

In the present study, we addressed the response of two human pathogens, *S. aureus* and *L. monocytogenes* to plectasin. These two pathogens differs in sensitivity towards plectasin with MIC values of 16-32mg/L for methicillin resistant *Staphylococcus aureus* (MRSA), and above 64mg/L for the less sensitive *Listeria monocytogenes* [6,7]. In addition, the two bacteria represent different routes of infection and may be exposed to different arrays of HDPs. *S. aureus* is a hospital- and community-acquired pathogen that causes a wide range of diseases including septicaemia, toxic-shock syndrome and food poisoning [8]. *S. aureus* is primarily extracellular and produces extracellular enzymes and toxins that cause damage to tissues. *L. monocytogenes* is a food borne pathogen causing gastroenteritis or septicaemia and meningitis in immunocompromised individuals [9]. As opposed to the infection mode of *S. aureus*, *L. monocytogenes* is an intracellular pathogen, able to spread from cell to cell within the host and thereby guarded against circulating immune factors.

The purpose of the present study was to investigate if resistance/tolerance towards plectasin could be induced in *S. aureus* and *L. monocytogenes* by transposon mutagenesis and if this resistance/tolerance would affect the mutants' response to other groups of antimicrobial peptides

Results

Plectasin does not cause cellular leakage

Many antimicrobial peptides affect the structural or functional integrity of the bacterial membrane, leading to pore formation and subsequently leakage of intracellular

components [10]. Therefore, we examined the extracellular protein-profile by SDS-PAGE analysis. When the two Gram-positive pathogens, *S. aureus* and *L. monocytogenes,* were grown with and without plectasin, there was no difference, indicating that the bacteria are not leaking macromolecules (data not shown). To support this notion, we determined the effect of plectasin on the membrane of the two species by measuring the amount of ATP leakage. In this study we also included three peptides representing each of the antimicrobial peptide groups: the plectasin-like defensin eurocin, the linear arginine-rich peptide protamine [11] and the α-helical peptide novicidin. ATP leakage profiles were similar for *L. monocytogenes* and *S. aureus* but differed between peptides. When either of the pathogens were exposed to the defensins, plectasin or eurocin, we found that the intracellular ATP concentration remained at the same level as the controls treated with peptide dilution buffer only, indicating that the defensins do not cause pore formation or membrane disruption of neither *L. monocytogenes* nor *S. aureus* (Figure 1). In contrast, protamine and novicidin resulted in increased ATP leakage thus suggesting that they are disrupting the membrane (Figure 1). Our finding is in agreement with previous results which showed slower killing kinetics for plectasin compared to membrane-perturbing compounds, indicating that plectasin does not function by perturbing the membrane [6].

Since the antimicrobial activity of plectasin does not involve membrane disruption we set out to search for an alternative mode of action. An increasing amount of evidence establishes that HDPs can have cytoplasmic and intracellular targets [12]. One such target is the bacterial DNA. However, we were unable to demonstrate binding of neither plectasin nor eurocin to DNA when examined by in vitro gel retardation (data not shown).

Identification of genes providing increased tolerance to Plectasin

In order to identify genes involved in the bacterial susceptibility towards plectasin, we created transposon mutant libraries in *S. aureus* 8325-4 and *L. monocytogenes* 4446 using *bursa aurealis* and Tn*917*, respectively. MIC values on agar plates were determined for the two wild types and the two transposon libraries were subsequently screened on plectasin-concentrations corresponding to 4, 5 or 10 fold MIC. Screening of the *S. aureus* mutant library resulted in identification of seven colonies with increased tolerance. However, after screening 40,000 colonies of *L. monocytogenes* transposon mutants, we found no mutants with increased tolerance. Both *S. aureus* and *L. monocytogenes* wild types were also screened on similar agar plates with plectacin and no spontaneous mutations, leading to changes in sensitivity, occurred.

Sequence analysis of two of the *S. aureus* mutants, revealed that the transposon element had inserted into the heme response regulator *hssR* that together with *hssS* forms a two component system (TCS) [13]. *S. aureus* require iron, and during infection, it can obtain iron through the haemolysin-mediated rupture of erythrocytes [15]. While heme is an important source of iron, high concentrations are toxic to *S. aureus* due to the molecule's reactivity [16]. Therefore, the HssRS TCS is able to sense high concentrations of heme and induces the expression of the HrtAB efflux pump that protects the cells against heme-mediated cell damage ([16,17].

No mutants were obtained from the screening of the transposon mutant library of *L. monocytogenes* for altered tolerance to plectasin. However, an orthologous system in *L. monocytogenes* LO28 was identified by homology search and we found that the response

regulator RR23 has a higher identity (48%) to HssR compared to other response regulators (30-35%) from *L. monocytogenes* LO28. In addition, RR23 have 99% identity to *L. monocytogenes* EGD-e lmo2583, previously suggested to be an *hssR* orthologoue [13]. To evaluate the importance of HssR on sensitivity to plectasin of another bacterium than *S. aureus*, a *L. monocytogenes* RR23 mutant was included in the experiments.

HssR modulates tolerance to defensins

In order to validate the phenotypes obtained by our *S. aureus* transposon mutant 8325-4 *hssR*::bursa, we transduced the transposon element to *S. aureus* 8325-4 wild type, giving the mutant 8325-4 *hssR*. In addition, we included another *S. aureus* wild type, *S. aureus* 15981, and the 15981-ΔTCS15 (15981- ΔTCS15 hssRS), which harbour a deletion of both the response regulator *hssR* and the histidine kinase *hssS* [18].

The Minimal Inhibitory Concentrations (MIC) for plectasin was determined for all the strains using the microbroth dilution method (Table 1) and a mutation in the *hssR* response regulator in *S. aureus* lead to a 2 to 4 fold increased tolerance compared to the wild type, regardless of the genetic background. This is in agreement with the initial finding, where we used 4 fold MIC in the plate screen for transposon mutants. The deletion of the *rr23* in *L. monocytogenes* had no effect on the tolerance towards plectasin (Table 1).

In addition, we tested whether the two-component system is involved in altered sensitivity to other antimicrobial peptides namely novospirin (a cathelicidin), novicidin (a cathelicidin), protamin (a linear peptide) and eurocin (a plectasin-like defensin). The *S. aureus*

hssR/*hssRS* mutants were also more tolerant to eurocin, the only other defensin, but were not altered in sensitivity to other groups of peptides (Table 1).

The ability of the *S. aureus hssR* mutants to cope with higher concentrations of the peptide compared to the wild type was confirmed in a growth experiment. The strains were grown with (concentrations known to inhibit growth) or without plectasin and the wild type did not grow in the presence of plectasin, but the response regulator mutants all grew (Figure 2). The growth experiment also showed that the mutant and wild type strains have similar growth kinetics when grown in TSA (Figure 2).

Both HrtAB and HssRS are required for Staphylococcal growth in hemin [13]. When we examined the growth of the *hssR* mutant compared to the wild type we also found it to be almost completely inhibited by 4 µM hemin, regardless of the presence or absence of plectasin (Figure 3). The expression of *hrtAB* efflux system has previously been shown to increase 45 fold by exposure to hemin through transcriptional activation by HssR [19]. However, a northern blot revealed that the expression of *hrtB* and *hssR* are not changed by addition of plectasin (data not shown).

Stress and antibiotic resistance of *hssR* mutant cells

The relatively small number of bacterial TCSs in *S. aureus* and *L. monocytogenes*, imply that some of them are able to sense several different stressors. In *Streptococcus pyogenes* the TCS CovRS, sense both iron starvation, antimicrobial peptides and several other stressors [20]. We have found that HssR sense defensins in addition to heme concentrations, we therefore determined if the HssRS two-component system affects

susceptibility to other types of stress. However, when the *S. aureus* and *L. monocytogenes* wild types and mutants were subjected to a variety of stress-conditions; growth at 15°C, 30°C, 37°C or 44°C, or growth with the addition of 4% NaCl, we found no difference in growth between the wild types and their respective mutants. We also examined the sensitivity of wild type and mutants to several antibiotics, i.e. ampicillin, gentamicin, sulfa/trimethoprim, rifampicin, tetracycline, amoxy/clavulan, cephalotin, clindamycin, enrofloxacin, fusidic acid and oxacillin and no change in MIC values were observed when the wild type *S. aureus* and *L. monocytogenes* and the corresponding response regulator mutants were compared (data not shown). Thus, as opposed to the CovRS TCS, HssR/RR23 from *S. aureus* and *L. monocytogenes* do not seem to sense other types of stress. The results for RR23 correspond with previous experiments, showing no stress phenotype for a *rr23* mutant [21].

Discussion

In the present study, we investigated how the antimicrobial peptide, plectasin, affects two human pathogens. Our results indicate that plectasin and another defensin, eurocin, do not perturb the *S. aureus* and *L. monocytogenes* membrane, but affect the bacterial survival differently. These results are in agreement with previous results that demonstrated slower killing kinetics of plectasin compared to known membrane pertubing compounds, which indicates that this defensin does not mediate cellular leakage [6]. However, the non-defensins, novicidin and protamin did lead to increased leakage, implying that the antimicrobial activity of these peptides involves disruptions of the bacterial membranes (Figure 1).

To identify genes involved in tolerance to plectasin, we screened transposon mutant libraries of *L. monocytogenes* and *S. aureus*. We were unable to identify any *L. monocytogenes* mutants more tolerant to the peptide compared to wild type. The *L. monocytogenes* wild-type is more tolerant to plectasin (MIC >64 µg/ml) compared to the *S. aureus* wild type (MIC = 8-16 µg/ml) [6,7, this work], which might explain the difficulties in obtaining less sensitive *L. monocytogenes* mutants. Seven *S. aureus* mutants, more tolerant to plectasin, were isolated. Two of these had the transposon inserted into the response regulator *hssR*, which is part of a two-component system (TCS), HssRS, involved in sensing heme concentrations [13]. A primary mechanism by which bacterial cells respond to changes in the environment is through the action of TCSs. TCSs typically consist of a membrane-bound histidine kinase that senses the changes and undergo autophosphorylation followed by transfer of the phosphoryl group to the regulator [22]. During contact with a host, *S. aureus* acquire heme as iron source, but surplus heme can be toxic. The HssRS system is important for sensing the level of heme, and for activating the ABC transporter system HrtAB, which protects the bacteria against heme-mediated damage [16,17]. The HssR response regulator seems to be important for *S. aureus* to sense its environment [13]. Changes in iron availability are an environmental signal indicative of mammalian host-pathogen interaction [23]. Our results reveal that a mutation in *hssR* increases the tolerance of *S. aureus* to two defensin-like HDPs, suggesting that the mutation of *hssR* lead to enhanced bacterial resistance to immune clearance. Defensins are an important part of the mammalian immune response and mutant pathogens, more susceptible to HDPs, are often attenuated in virulence, indicating that the intrinsic resistance to HDPs plays a key role in bacterial infection [24]. It has previously been reported that a mutation in *S. aureus hssR* leads to increased virulence [13]. In

addition, *hssR* mutants have been shown to affect the innate immune response against *S. aureus* infection. The deletion of *hrtA* increases the expression of immunomodulatory factors and it has been suggested that growth in heme, could be sensed as a physiological signal for invading *S. aureus* to switch from a cytolytic toxin secretion profile to an immunoevasive response [13]. This hypothesis could explain why the *hssR* mutant survive plectasin better compared to the wild type and this ability to cope with defensins, could be an important part of the increased virulence observed. Recently, a link between iron starvation and HDP resistance in *Yersinia pseudotuberculosis* has been shown, supporting the idea that bacteria can sense that they are inside a host and then coordinate their response accordingly [25].

We did not observe an upregulation of neither *hssR* nor *hrtA* when *S. aureus* is exposed to plectasin. Previous results have shown a 45 fold upregulation of *hrtAB* when exposed to exogenous hemin [19]. The lack of plectasin regulation of the systems implies that the two-component system do not sense the defensins and the ABC transporter system HrtAB is not involved in exporting the peptides. Instead, the deletion of *hssR* affects the production and secretion of various virulence factors, including proteins that can neutralize peptides.

A possible HssR homologue, RR23, exist in *L. monocytogenes*. However, a mutation in this response regulator showed no change in growth or survival when exposed to the peptides and previous results have shown that the RR23 mutant is not important for virulence [21]. Homologues of the HrtAB and HssRS systems are found conserved across Gram-positive pathogenic bacteria, including *L. monocytogenes* [13, this work]. With the results presented here, it can be argued that the primary function of the systems, is to

respond to heme exposure in the Gram-positive species, but the ability to produce immune evasive factors and enhance its resistance to immune clearance is only found in *S. aureus* and not in *L. monocytogenes*. Whether the difference in sensitivity towards plectasin between *L. monocytogenes* and *S. aureus* can be explained by the variations in virulence factors and different routes of infection of the two pathogens remains elusive.

Conclusion

We found that the *S. aureus* response regulator HssR, but no the corresponding RR23 from *L. monocytogenes*, is involved in the organisms' sensitivity to defensins, exemplified by plectasin. Mutating the *hssR* leads to increased tolerance towards plectasin and eurocin. The HssRS two component system have previously been shown to be important for heme homeostasis and an *hssR* mutation leads to increased virulence [13]. Taken together these results further indicate the importance of this system in sensing environmental cues and responding accordingly. This result support the notion that the system is able to sense internal host tissue and shift to an immune evasive response and that the mutation in *hssR* leads to enhanced bacterial resistance to host immune factors. During the course of infection, the bacteria must not only cope with iron starvation but also resist antimicrobial peptides, including defensins. Whether the difference in responding to the HDPs between *L. monocytogenes* and *S. aureus* is due to the differences in infection processes still remains unclear, but our results indicate a functional difference between RR23 and HssR and the genes regulated by these regulators, which might explain the difference in HDPs susceptibility between the two strains.

Methods

Strains and culture conditions

Listeria monocytogenes strain 4446 [26]. *L. monocytogenes* LO28 [27], *L. monocytogenes* LO28 RR23 [21]. *S. aureus* 8325-4 ([28]. 8325-4 *hssR*::bursa (this work), 8325-4 *hssR* (this work), 15981 [30], 15981 ΔTCS15 [18] in this paper referred to as 15981ΔTCS15 *(hssRS).* The bacteria were grown in Brain Heart Infusion broth (BHI, CM0225 Oxoid) (*L. monocytogenes*) or Tryptone Soy Broth (TSB, CM0129 Oxoid) (*S. aureus*). When appropriate, antibiotics were added at the following concentrations erythromycin 5 (*L. monocytogenes*) and 10 µg/ml (*S. aureus* 8325-4), chloramphenicol 10 µg/ml and tetracycline 12,5 µg/ml (Sigma).

Host defense peptides.

Protamine was purchased from Sigma (P4020-5G). Plectasin, eurocin, novicidin, and novispirin G10 were supplied by Department of Antiinfective Discovery, Novozymes A/S. The antimicrobial peptides were dissolved in 0.01% acetic acid/0.1% bovine serum albumin (Sigma, A7906).

Determination of the effect of Plectasin on the bacterial envelope - ATP measurements.

L. monocytogenes and *S. aureus* were grown in TSB at 37 ℃. Bacteria were harvested (10 min at 2,000 × g) at mid-exponential phase (OD_{546} of 2.5 and 1.0 for *S. aureus* and *L. monocytogenes*, respectively), washed once in 50 mM potassium phosphate buffer pH 7.0 and once in 50 mM HEPES buffer pH 7.0. The pellet was resuspended in 50 mM HEPES pH 7.0 to a final OD_{546} of 10. Bacteria were stored on ice and used within 5 hours. Bacteria were energized in 50 mM HEPES (pH 7.0) with 0.2% (wt/vol) glucose and treated with 500

µg/ml plectasin or eurocin. ATP was determined using a bioluminescence kit (Sigma, FLAA-1KT) and a BioOrbit 1253 luminometer. Total ATP content was determined by rapidly permeabilising 20 µl cell suspension with 80 µl dimethyl sulfoxide. The cell suspension was diluted in 4.9 ml sterile water, and ATP content was determined in 100 µl of the preparation as described by the manufacturer. To determine the extracellular ATP concentration, the 20 µl cell suspension was mixed with 80 µl sterile water and analyzed as described above. Intracellular ATP concentrations were calculated by using the intracellular volumes of 0.85 and 1.7 μm^3 for *S. aureus* and *L. monocytogenes*, respectively. The number of cells in suspension was determined by plate spreading.

Extracellular protein

Prewarmed TSB/BHI (25 ml) in a 250 ml Erlenmeyer flask was inoculated with and without plectasin and incubated with shaking at 37°C overnight (≈ 17 h). The next morning, the exact OD_{600} ml^{-1} of the cultures was measured, and 15 ml of culture was centrifuged to precipitate the cells. The supernatant was transferred to a 50 ml Blue cap bottle (placed in an ice/water bath), and the extracellular proteins were precipitated by adding one volume of ice-cold 96% EtOH and left in the refrigerator overnight for proteins to precipitate. Precipitated proteins were collected by centrifugation (15,000 x *g*; 30 min; 0°C). Protein pellets were suspended in a volume of 50 mM Tris-HCl (pH) adjusted to the original OD_{600} ml^{-1} of the overnight culture so that 15 ml of overnight culture with OD_{600} ml^{-1}= 5.0 was suspended in 0.8 ml of 50 mM Tris-HCl (pH). A sample of 15 µl of the protein extracts was analysed on NuPAGE® 4–12% Bis-Tris gels (Invitrogen) using the X Cell SureLock® Mini-Cell system (Invitrogen) as recommended by the supplier. The gels were Coomassie stained using GelCode® Blue Stain Reagent (Pierce).

DNA-binding analysis

Gel retardation analysis were performed by mixing 100 ng of plasmid DNA (pBluescript II SK^+) with increasing amounts of peptide in 20 µl binding buffer (5% glycerol, 10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCL and 50 µg ml^{-1} bovine serum albumin). Reaction mixtures were incubated 1 h at room temperature. Subsequently, 4 µl loading buffer (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA) (Fermentas) and a 12 µl aliquot was subjected to 1% agarose gel electrophoresis in 0.5 × Tris-acetat-EDTA (40 mM Tris acetate and 1 mM EDTA, pH 8.0).

Transposon library in *L. monocytogenes* and *S. aureus*.

Transposon mutagenesis of *L. monocytogenes* 4446 was performed with the temperature-sensitive plasmid pLTV1 [30] as described, but with modifications. *L. monocytogenes* 4446 harbouring pLTV1 was grown overnight at 30°C in BHI containing 5 µg/ml erythromycin. The bacterial culture was then diluted 1:200 in BHI erm_5 and grown for 6 h at 42°C. Aliquots were plated onto BHI erm_5 plates and incubated at 42°C. Colonies were harvested from the plates in BHI and stored in 30% glycerol at -80 °C. To determine the transposition frequency, the transposon library was plated onto BHI containing 5 µg/ml erythromycin. 100 colonies were picked and streaked onto BHI plates containing 5 µg/ml erythromycin, 10 µg/ml chloramphenicol, and 12.5 µg/ml tetracycline, respectively, and incubated at 30 °C for 48 h. The transposition frequency was calculated as the percentage of colonies growing only on BHI + 5 µg/ml erythromycin and BHI+10 µg/ml chloramphenicol (harbouring only the transposon) but not on BHI+12.5 µg/ml tetracycline

(still harbouring the plasmid). Transposon mutagenesis of *S. aureus* 8325-4 with bursa aurealis was performed as described [31].

Screening of transposon library for plectasin tolerant mutants

The transposon mutant libraries were screened on agar plates for increased tolerance to plectasin as compared to wild-type sensitivity. Wild-type sensitivity was determined by plating approx. 1.0×10^7 CFU/ml on TSA containing plectasin (*S. aureus*) and approx. 1.0×10^5 CFU/ml on Muller Hinton Broth agarose plates (MHB, 212322 Becton Dickinson) with plectasin (*L. monocytogenes*). Plates were incubated at 37°C for 3 days and inspected for growth. The transposon libraries were screened on TSA with 300 µg/ml, 500 or 750 µg/ml plectasin (*S. aureus*) or MHB plates with 250 µg/ml or 500 µg/ml plectasin (*L. monocytogenes*) at 37°C for up to 7 days.

Determination of Minimum Inhibitory Concentrations (MIC) of antimicrobial peptides in liquid medium.

Minimal inhibitory concentrations (MIC) of plectasin, eurocin, protamine, novicidin, and novispirin G10 were determined using a microbroth dilution method [32]. Colonies from a BHI plate incubated overnight at 37ºC were suspended in MHB pH 7.4 to a turbidity of 0.11-0.12 at 546 nm (approx. 1.0×10^8 CFU/ml) and diluted in MHB to a concentration of 5.0×10^5 CFU/ml. Ninety µl of bacterial suspension was incubated with 10 µl of peptide solution in polypropylene 96-well plates (Nunc, 442587) for 18-24 h at 37 °C. The peptide solutions were made fresh on the day of assay. The range of concentrations assayed were 0.25–256 µg/ml for plectasin and eurocin, 0.125-128 µg/ml for protamin and novispirin G10,

and 0.031-32 µg/ml for novicidin. MIC was the lowest peptide concentration at which visual growth was inhibited.

Influence of hemine and plectacin on growth of *S. aureus*

Overnight cultures of *S. aureus* were diluted to OD_{600} = 0.05 in TSB with and without 4 µM hemin and/or plectasin. Growth at 37C. OD_{600} measurements were made every 30 minutes.

Potential influence of plectacin on *hssR* expression. Wild type *S. aureus* and the *hssR* mutant were grown at 37°C with vigorous shaking and at an optical density at 600 nm (OD_{600}) of 0.45 ± 0.1 samples were withdrawn for the isolation of RNA. Then 35µg/ml Plectasin was added to the growing culture, and after 10 and 80 minutes samples were also withdrawn. Cells were quickly cooled on ice bath and frozen at –80°C until extraction of RNA. Cells were lysed mechanically using the FastPrep machine (Bio101; Q-biogene), and RNA was isolated by the RNeasy mini kit (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions. Total RNA was quantified by spectrophotometric analysis (λ = 260 nm), and 5 µg of RNA of each preparation was loaded onto a 1% agarose gel and separated in 10 mM sodium phosphate buffer as described previously [33]. RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by capillary blotting as previously described [34]. Hybridization was performed using gene-specific probes that had been labeled with [^{32}P]dCTP using the Ready-to-Go DNA-labeling beads from Amersham Biosciences [35]. Internal fragments of the genes were used as templates in the labeling reactions. Primers for internal fragments : hrtB-1 5´CACTCAATAAATGTCTTGTC 3´, hrtB-2 5´AAGGTAATTCATCAAGAACC 3´, hssR-1

5´AATGTCTTGTTGTCGATGAC 3´ , hssR-2 5´ TTATAGCCTTGTCCTCTTAC 3´. All steps were repeated in two independent experiments giving similar results.

Stress and antibiotic resistance of *S. aureus and L. monocytogenes*

Cultures were grown exponentially in TSB/BHI at 37°C. At OD_{600} = 0.2, the cultures were diluted 10^{-1}-, 10^{-2}-, 10^{-3}- and 10^{-4}-fold, and 10 µl of each dilution was spotted on TSB/BHI plates. The plates were incubated at the indicated temperatures. In addition plates containing 4% NaCl were spotted and incubated in a similar way.

Antimicrobial susceptibility to ampicillin, gentamicin, sulfa/trimethoprim, rifampicin, tetracycline, amoxy/clavulan, cephalotin, clindamycin, enrofloxacin, fusidic acid and oxacillin was performed with a commercially available MIC technique using dehydrated antimicrobials in microtitre wells (Trek Diagnostic systems Ltd., UK).

Authors´ contributions

LET participated in the design of the study, did the *S. aureus* transposon mutant library, growth analysis, stress and antibiotic analysis, northern blot, mutant creation and drafted the manuscript, CTG did the *L. monocytogenes* transposon mutant library, carried out the screening, MIC determinations and ATP leakage analysis, participated in the design of the study and helped revising the manuscript. SG did growth experiments with and without plectasin and hemin and DNA binding analysis. TTW screened the *S. aureus* transposon library and identified the *hssR* gene, HHK supplied the peptides, plectasin, eurocin, novicidin, and novispirin G10. LG participated in the design of the study and helped revising the manuscript. HI participated in the design of the study and helped revising the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1 - Measurement of ATP leakage from *Staphylococcus aureus* after treatment with plectasin (A), eurocin (B), protamine (C), and novicidin (D).

Measurement of intracellular (IC) and extracellular (EC) ATP after treatment with plectasin (500 µg/mL), eurocin (500 µg/mL), protamine (1,000 µg/mL), novicidin (1,000 µg/mL), or peptide dilution buffer. Treatment with the two defensins does not lead to leakage of intracellular ATP, whereas treatment with protamine and novicidin lead to leakage of ATP. Representative results from *S. aureus* are shown as treatment of *S. aureus* and *L. monocytogenes* resulted in similar leakage profiles. Symbols are averages of duplicate, independent determinations and errorbars represent standard deviations.

Figure 2 - Growth of *Staphylococcus aureus* wild-type and *hssR* mutants in the presence of plectasin.

Plectasin inhibited the growth of *S. aureus* 8325-4 and 15981 wild-types but hardly affected the growth of the 8325-4 *hssR*::bursa transposon mutant, the transduced 8235-4 *hssR* mutant or the 15981 ΔTCS15 (*hssRS*) mutant.

Figure 3 - Growth of *Staphylococcus aureus* wild-type and *hssR* mutants in the presence of hemin and plectasin.

The growth of the *S. aureus* 8325-4 wild-type is only affected by plectasin and not hemin. On the contrary, the 8325-4 *hssR* mutants do not grow in the presence of hemin, regardless of the presence or absence of plectasin, confirming the heme-sensitive phenotype of *hssR* mutants.

Tables

Table 1 - MIC values of host defence peptides (HDPs) against *S. aureus* and *L. monocytogenes* wild-types and two-component system mutants. Plec: plectasin, Euro: eurocin, Prot: protamine, NovC: novicidin, NovS: novispirin.

Figure 1

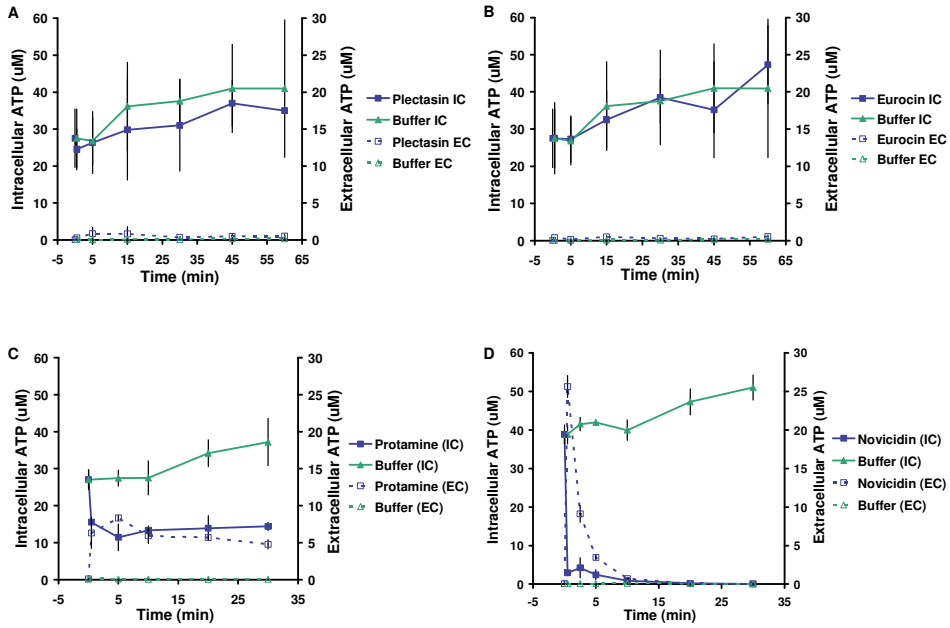
A
Intracellular ATP (uM)
Extracellular ATP (uM)
Time (min)
Plectasin IC
Buffer IC
Plectasin EC
Buffer EC
B
Intracellular ATP (uM)
Extracellular ATP (uM)
Time (min)
Eurocin IC
Buffer IC
Eurocin EC
Buffer EC
C
Intracellular ATP (uM)
Extracellular ATP (uM)
Time (min)
Protamine (IC)
Buffer (IC)
Protamine (EC)
Buffer (EC)
D
Intracellular ATP (uM)
Extracellular ATP (uM)
Time (min)
Novicidin (IC)
Buffer (IC)
Novicidin (EC)
Buffer (EC)

Figure 2

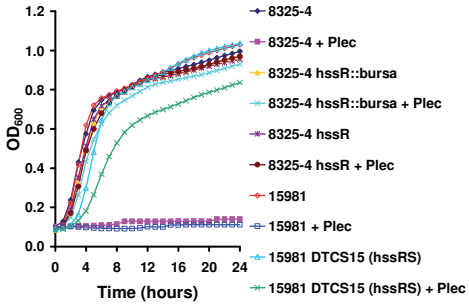
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600
0
4
8
12
16
20
24
Time (hours)
8325-4
8325-4 + Plec
8325-4 hssR::bursa
8325-4 hssR::bursa + Plec
8325-4 hssR
8325-4 hssR + Plec
15981
15981 + Plec
15981 DTCS15 (hssRS)
15981 DTCS15 (hssRS) + Plec

Figure 3

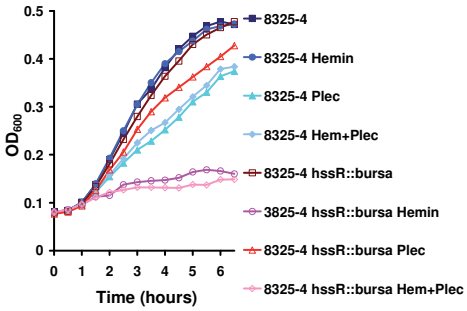
0.5
0.4
0.3
0.2
0.1
0
OD600
0 1 2 3 4 5 6
Time (hours)
8325-4
8325-4 Hemin
8325-4 Plec
8325-4 Hem+Plec
8325-4 hssR::bursa
3825-4 hssR::bursa Hemin
8325-4 hssR::bursa Plec
8325-4 hssR::bursa Hem+Plec

Table 1

Strain	Description	MIC (μg/ml)				
		Plec	Euro	Prot	NovC	NovS
8325-4	*S. aureus* wild-type	16	32	16	1	128
8325-4 *hssR::bursa*	Transposon mutant	32	64	16	1	128
8325-4 *hssR*	Transduced 8325-4 *hssR* mutant	32	64	16	1	128
15981	*S. aureus* wild-type	8	8	16	1	>128
15981 *ΔTCS15 (hssRS)*	*hssRS* deletion mutant	32	32	16	1	>128
LO28	*L. monocytogenes* wild-type	64	128	16	1	16
LO28 RR23	*rr23* insertion mutant	64	128	16	1	16

Paper 4

Anne Holch¤, Caroline Trebbien Gottlieb¤, Marianne Halberg Larsen, Hanne Ingmer, Lone Gram (2009).

A group of food processing persistent *Listeria monocytogenes* strains are poor invaders in trophoblastic cells but form normal plaques in fibroblastic cells despite *actA* deletion.

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¤ A.H. and C.T.G. contributed equally to this study.

A group of food processing persistent *Listeria monocytogenes* strains are poor invaders in trophoblastic cells but form normal plaques in fibroblastic cells despite *actA* deletion

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25th November 2009

Running title: Invasion and plaque formation of *Listeria monocytogenes* strains

Abstract

We have previously shown that a group of genetically similar *Listeria monocytogenes* strains (molecular sub-type RAPD 9) that can persist in fish processing are low virulent in simple eukaryotic models but one of these strains was highly virulent against guinea pig fetuses. The purpose of this study was to determine if the persistent RAPD 9 strains have a preference for the placenta and therefore pose a particular risk to unborn fetuses. The RAPD 9 strains were compared to clinical strains from maternofetal cases with respect to phenotypic behavior in cell line systems mimicking two of the steps in crossing of the placental barrier. Additionally, three key virulence genes, *inlA*, *inlB*, and *actA*, were sequenced to determine if there was a correlation between cell invasion, genotype and the type of infection. The RAPD 9 strains invaded human placental trophoblasts less efficiently than other *L. monocytogenes* strains, and this could be explained by the presence of a premature stop codon in *inlA* from the RAPD 9 strains. In addition, they did not show enhanced intracellular replication in these trophoblasts. Likewise, the RAPD 9 strains did not have an increased ability to spread from cell to cell in mouse fibroblasts. Interestingly, 8 of 15 strains, including the RAPD 9 strains and maternofetal strains, had a 105 nucleotide deletion in *actA*, but this did not affect the cell-to-cell spread. We conclude that the RAPD 9 strains can still be regarded as low-virulent with respect to human listeriosis and do not pose a particular increased risk to pregnant women.

Words: 254

Introduction

Listeria monocytogenes is a gram positive pathogenic bacterium that can cause food-borne listeriosis. It is a ubiquitous environmental bacterium, and it is therefore continuously introduced to the food processing plants through contaminated raw material. Once introduced, some molecular sub-types are able to persist in the food processing plants despite thorough cleaning and disinfection procedures (1,32,39,48). Such persistent strains are likely to contaminate the food products and may be the cause of food borne infections (35).

We have shown that specific molecular subtypes of *L. monocytogenes* can persist for years in the seafood processing environment (51) and since these strains are likely food contaminants, it is important to determine their virulence potential. We found that strains representing a particular prevalent persistent molecular sub-type (the so-called RAPD type 9 (Random Amplified Polymorphic DNA)), had a lower virulence potential than clinical strains in a number of simple eukaryotic models, e.g. invasion into Caco-2 cells (14). However, in a more complex biological model using pregnant guinea pigs, a RAPD type 9 strain surprisingly infected the placentas and fetuses just as efficiently as a clinical strain (15). We therefore hypothesized that this specific sub-type may have an altered (enhanced) ability to cross the placental barrier for instance by an enhanced invasion into placental cells or an enhanced ability to intracellular spread.

During listeriosis, *L. monocytogenes* disseminates in the blood after crossing the intestinal barrier. From the maternal blood *L. monocytogenes* must cross the placental tissue to gain access to the fetal blood stream. The placenta is composed of interlocking maternal and fetal tissues and is formed when fetal epithelial cells, trophoblasts, proliferate into the uterus wall (19,27). Thus, *L. monocytogenes* from the maternal blood must first invade the fetally derived trophoblasts and spread through the connective tissue before it can cross the endothelial cells of the fetal blood

vessels and infect the fetus. A number of virulence factors are important for the fetal infection by *L. monocytogenes*, and mutations in these key virulence genes lead to less virulent *L. monocytogenes* (9). The surface proteins InlA and InlB interact with their respective receptors, E-cadherin (28) and Met, gC1qR or proteoglycans (4,17,41) and mediate internalization of *L. monocytogenes* into non-phagocytic cells. InlA is involved in invading intestinal epithelial cell lines (10,25) and has also been shown to be important for invasion of human trophoblastic cell lines as well as human primary trophoblastic cultures and placental explants (2,12,26). In contrast, *in vivo* studies on pregnant guinea pigs and mice showed that InlA was not important for fetoplacental invasion (2,23). InlB appears not to be essential for *in vitro* infection of placental cells (2, 26). However, the interaction of both InlA and InlB with their respective receptors is species-specific, and thus the important role that these proteins play in crossing of host barriers, can only be studied in species permissive to both these pathways. Recently Disson et al. (9) have shown an interdependent role of InlA and InlB for crossing the blood-placenta barrier *in vivo* in both knock-in mice (hEcad) and gerbils, which like humans are permissive to both the InlA and the InlB pathways. Apart from the laboratory-generated *inlA* deletion mutants, field strains with mutations in *inlA* leading to premature stop codons (PMSC) have been detected in *L. monocytogenes* from France (34), USA (30) and Japan (11). These mutations lead to attenuation in invasion of intestinal epithelial cells (30,33,40), but it is not known if the invasion into trophoblasts is affected.

ActA is important for cell-to-cell spread (5), is involved in invasion into epithelial cells (45), and ActA-mediated cell-to-cell spread plays a major role in crossing the fetoplacental barrier in both a guinea pig and a mouse model (3,23). When inside the placental tissue, ActA is essential for further infection of the fetus (3). An identical deletion of 105 nucleotides, in the proline rich region of *actA* has been reported in several strains (29,44,50) and the influence of this deletion on the ability to form plaques is still unresolved. Neither Sokolovic et al. (44) nor Roberts & Wiedmann (37) could correlate the length of ActA and the ability of the bacterium to spread as indicated by its ability to form plaques.

The purpose of this study was to determine if the discrepancy between assessment of virulence potential of the processing plant persistent strains (RAPD 9) in gastro-intestinal cells and in the *in vivo* pregnant guinea pig model could be explained by systematic differences in invasion into and spread between cells and if systematic differences in virulence gene sequences could be the cause of such potential differences. We hypothesized that the RAPD type 9 strains would have a preference for the placenta and would thus pose a particular risk to unborn fetuses. To assess the virulence potential of the RAPD type 9 stains in maternofetal listeriosis further, we investigated the phenotypic behavior of these RAPD type 9 strains in model systems mimicking two of the steps in the crossing of the placental barrier. We compared this behavior to strains from clinical cases of listeriosis, especially maternofetal cases in their ability to invade and grow in a trophoblastic cell line, JAR, and to spread in a fibroblastic cell line, L929. In addition, *inlA*, *inlB* and *actA* which are the three virulence genes important for these steps were sequenced.

Materials and methods

Strains, culture conditions and characterization. Experiments were carried out with a culture collection of 17 *Listeria monocytogenes* strains representing different origins (food processing environment, food products and human clinical strains), RAPD-types, serotypes and lineages (table 1). Four strains (N53-1, La111, H13-1, M103-1) all represent a unique persistent sub-type (RAPD type 9) of *L. monocytogenes*. Strain La22 was isolated from a food sample but its RAPD type is similar to another RAPD sub-type that we have found as persistent in a fish processing plant (48). Stock cultures were stored at -80°C in 4% (w/v) glycerol, 0.5% (w/v) glucose and 2.0% (v/w) skimmed milk powder. The bacteria were grown in Brain Heart Infusion (BHI) broth (Oxoid, CM0225) and enumeration was done onto BHI agar (Oxoid, CM0225 and AppliChem, A7354). For

all cell assays, bacteria were grown at 37°C with aeration for 20 h. Serogrouping of the strains was done using Listeria O antisera Type 1 and 4 (Becton Dickinson, 223001 and 223011) according to the manufacturer, and lineage separation was done as in Wiedmann et al. (50). RAPD typing was done according to Vogel et al. (49).

Invasion in Caco-2 cells. The human colorectal adenocarcinoma cell line Caco-2 (HTB 37) was obtained from LGC standards and grown in Eagle's Minimum Essential Medium (MEM) (Invitrogen, 42360024) supplemented with 20% FBS (Lonza, DE14-830), 0.1 mM non-essential amino acids (Invitrogen, 11140035), and 25 µg/ml gentamycin (Gibco, 15750-037). Invasion was determined as described earlier (13).

Invasion and intracellular growth in JAR cells. The human choriocarcinoma cell line JAR (HTB-144) was obtained from LGC standards and grown in F12 (Lonza, BE12-615) supplemented with 10% Fetal Bovine Serum (FBS) (Lonza, DE14-830) and 25 µg/ml gentamycin (Gibco, 15750-037). For the invasion assay, the JAR cells were adjusted to 5×10^4 cells/ml in F12+10% FBS and grown in a 24-well tissue culture plate (TPP, Trasadingen, Switzerland) for 24 h at 37°C with 5% CO_2 to reach a monolayer. The bacterial overnight cultures were adjusted to 1×10^6 CFU/ml by dilution in F12+10% FBS. The cells were washed one time in saline water (0.9% NaCl, pH 7.2) and 1 ml of diluted bacteria was added to each well. Following, the plates were centrifuged for 5 min at 150 *x g*. After 1 h of incubation, the cells were washed in saline water and F12+10% FBS + 50 µg/ml gentamycin was added to each well, followed by a 1 hour incubation at 37°C. The cells were washed one time with saline water and lysed with 1 ml 0.1% TritonX-100. The number of bacteria released was expressed in CFU/ml by plating appropriate dilutions onto BHI agar plates. The ability of *L. monocytogenes* to grow intracellularly in JAR cells was studied for all 17 strains. The JAR cells was infected as described above, the monolayer was washed twice with saline water and the extracellular bacteria were killed by incubation with F12 +10% FBS + 50 µg/ml gentamycin for one hour at 37C. The media was removed and F12 +10% FBS + 25 µg/ml gentamycin were added

to each well. The plates were incubated for 2 h, 3.5 h or 5 h at 37°C. At each time the media was removed and cells were lysed and the number of intracellular bacteria was determined as described above. All experiments were carried out in duplicate in three independent trials.

Invasion and plaque formation in L929 cells. The mouse fibroblastic cell line L929 was obtained from European Collection of Animal Cell Cultures (ECACC, 85011425) and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, BE12-604F) with 10% (FBS) (Lonza, DE14-830) and 25 µg/ml gentamycin (Gibco, 15750-037). For the invasion assay, L929 cells were adjusted to 3×10^5 cells/ml in DMEM+10% FBS and grown in 24-well tissue culture plates (TPP, Trasadingen, Switzerland) for 24 h at 37°C with 5% CO_2 to reach a monolayer. The bacterial overnight cultures were adjusted to 5×10^4 CFU/ml by dilution in DMEM+10% FBS and invasion was determined as described for the JAR cells, except that L929 cells were incubated with DMEM + 10% FBS + 50 µg/ml gentamycin to kill extracellular bacteria prior to lysis. The detection limit with this setup was approx 1 CFU/ml. For the plaque formation assay, the L929 cells were grown in a 6-well tissue culture plate (TPP, Trasadingen, Switzerland) for 24 h at 37°C with 5% CO_2 to reach a monolayer. The bacterial cultures were adjusted to $5x10^4$ CFU/ml by dilution in DMEM with FBS. The L929 cells were washed two times in saline water and 3 ml of the diluted bacteria was added to each well. The plates were centrifuged for 5 min at 150 *x g*. After 1 h of incubation at 37°C, the L929 cells were washed in saline water, followed by addition of 2 ml overlay consisting of DMEM, 0.5% agarose, 10 µg/ml gentamicin. The wells were incubated 4 days at 37°C with 5% CO_2. The plaques were visualized by the addition of 2 ml DMEM containing 0.5% agarose and 0.01% neutral red (Sigma, N2889). The experiment was carried out in duplicate in three independent trials. To verify the assay, we included *L. monocytogenes* 10403S and 10403S delta *actA* (kindly provided by D. Portnoy, University of California) as a positive and negative control of plaque formation (data not shown). The plaque areas were measured using ImageJ software and plaque size was normalized to the size of the well. The measurement included 10 plaques per strain from one experiment.

Sequencing of virulence genes. Primers (DNA Technology, Århus, Denmark) of PCR amplification if *inlA, inlB* and *actA* were also used for sequencing of the purified PCR products (Table 2). The sequencing was done by DNA Technology (Århus, Denmark) and Eurofins MWG Operon (Ebersberg, Germany). To eliminate errors in the sequencing, each PCR product was sequenced with both the forward and reverse primer. The *inlA* and *inlB* genes were fully sequenced whereas the *actA* gene was sequenced partly.

Statistical analyses. The Mann-Whitney test was used to compare groups of full-length and deletion in *actA* with respect to either number of plaques or plaque sizes. The Kruskal-Wallis test was used to test for associations between invasive ability, number of plaques, and plaque sizes between groups of strains of different origin. All analyses were performed using GraphPad Prism statistical software.

Nucleotide sequence accession numbers. The *inlA*, *inlB* and *actA* gene sequences have been deposited in GenBank under the accession numbers xxx to xxx and GU079614 to GU079626 and GU060665 to GU060678, respectively.

Results

RAPD type 9 strains have a low invasive ability in placental trophoblasts. The ability of *L. monocytogenes* strains (RAPD type 9 and others) to invade placental trophoblasts was examined in the choriocarcinoma cell line JAR. The group of persistent RAPD type 9 strains invaded the trophoblasts at a significantly lower level than the maternofetal strains did ($P < 0.05$), but also EGD and LO28 displayed low invasion in JAR cells (Figure 1). Since *L. monocytogenes* invades placental trophoblasts in an InlA-E-cadherin dependent manner (26), the observed lower invasion

of the RAPD type 9 strains and LO28 in JAR cells is supported by the presence of premature stop codon in *inlA* from these strains (see below).

The ability to invade trophoblastic cells of the two lineage groups, lineage I and lineage II was compared and lineage I strains invaded to a significantly higher level than lineage II strains ($P < 0.05$). The two strains derived from food samples, 7418 and La22, also invaded the trophoblasts in a very high level, comparable to the maternofetal strains. The high invasion of the maternofetal and human clinical strains was also seen in Caco-2 cells (Table 1).

All strains have similar growth rates in JAR cells. We examined intracellular growth in JAR cells to determine if the RAPD type 9 strains were able to multiply to higher numbers once inside the placental cells. There was however no difference between the RAPD type 9 strains and the other strains (results not shown) indicating that the RAPD type 9 strains have no intracellular growth benefits compared to the clinical strains.

Differences in the ability of cell-to-cell spread in L929 cells. ActA is involved in cell-to-cell spread by inducing the polymerization of actin thus propelling *L. monocytogenes* through the host cell cytoplasm and into neighboring cells resulting in plaque formation. The ability of the strains to spread from cell to cell was evaluated in a plaque assay in the mouse fibroblastic cell line L929 (Figure 2). All strains invaded the L929 cells in a similar level (Table 1) and all strains were able to form plaques in L929 cells (Table 1 and Figure 2).

None of the four groups formed plaques significantly different from each other ($P > 0.05$), especially the group of persistent RAPD type 9 strains did not form a higher number of plaques as an indicator of increased cell-to-cell spread (Figure 2) . One maternofetal strain (3272-03) showed an extreme ability to form plaques as the number of plaques was more than double as all the numbers of the other strains. We did not find differences between the size of the plaques when

strains were grouped according to origin ($P > 0.05$), or presence of *actA* deletion ($P > 0.05$) (see below), however lineage I strains formed significantly larger plaques as compared to lineage II strains ($P < 0.05$).

RAPD type 9 strains harbor important mutations in *inlA* and *actA*. We have sequenced the three virulence genes, *inlA*, *actA* and *inlB* from all 15 wild type strains used in this study, because these three genes are important for invasion and the intracellular lifestyle of *L. monocytogenes*. In a previous study, we found that strains belonging to the food processing persistent RAPD type 9 group had a low ability to invade into the intestinal epithelial cell line Caco-2 (13,14), and we hypothesized that single point mutations in *inlA* could cause this result. In the present study we were able to identify the presence of a premature stop codon (PMSC) in all the four RAPD type 9 strains. We identified a nucleotide substitution from cytosine to a thymidine at position 1474, resulting in a stop codon at position 492 (Figure 3A), leading to export of InlA instead of incorporation into the cell membrane (18,33). LO28 was the only other strain containing PMSC, which is already known. The maternofetal strain 4810-98 had a small deletion of 9 nucleotides starting at position 2214 (result not shown). This deletion did not influence the ability of this strain to invade trophoblasts. The deletion was present at the membrane anchoring region of InlA and might therefore not influence the activity of InlA.

Three of 15 strains contained a complete *inlB* sequence, but in 12 strains we detected two nucleotide substitutions; one from guanine to adenine at position 350 resulting in an amino acid substitution from alanine to threonine (position 117) and one from guanine to adenine (position 395) leading to an amino acid substitution from valine to isoleucine (position 132) (Figure 3B). The first mutation was seen in the four RAPD type 9 strains, two maternofetal strains (12443 and 3272) and one food strain (La22). The other type of mutation was seen in all of the strains except EGD, LO28 and the maternofetal strain 3495-04. None of the strains contained any nonsense mutations in *inlB*.

When *actA*, encoding actin-polymerizing protein essential for intracellular motility and cell-to-cell spread was sequenced, a deletion of 105 nucleotides (35 amino acids) was seen in eight out of the 15 strains (Figure 3C). The deletion is present at amino acid 305 to amino acid 340. This deletion is in the region encoding the central region of proline-rich repeats required for binding to the vasodilator-stimulated phosphoprotein (VASP) (7,22). The deletion was not only seen in the four RAPD type 9 strains, but also in four of the clinical strains, of which two have caused fetal infection. This suggests that the deletion in the *actA* repeat region does not influence the ability to cause fetal infection. Also, this deletion did not influence the number of plaques formed ($P > 0.05$) (Table 1).

Discussion

In this study, we have addressed the apparent discrepancy between the virulence potential of genetically similar food processing persistent *L. monocytogenes* strains (RAPD type 9 strains) as they were low virulent in simple eukaryotic models (13) but one of these strains was highly virulent in the complex pregnant guinea pig model (15). The persistent RAPD type 9 strains have not been implicated in listeriosis outbreaks in Denmark (Birgitte Smith, unpublished results) but other persistent *L. monocytogenes* have been the cause of outbreaks, e.g. in the US (35,36). This emphasizes the need for a more thorough understanding of the physiology of these persistent strains to provide adequate insight about the virulence of field isolates and the likelihood that a given strain will cause illness.

We hypothesized that the disagreement between virulence potential in different models could be explained by an increased ability of the RAPD type 9 strains to execute one (or several) of the

infectious steps involved in crossing the placenta membrane from the maternal to the fetal bloodstream. To investigate this, we studied the phenotypic behavior in cell line models and sequenced important virulence genes (*inlA*, *inlB* and *actA*) from a genetically similar group of persistent strains and compared this to human clinical strains including strains isolated from maternofetal listeriosis.

The InlA receptor E-cadherin is present on the cell wall of trophoblast cells and is responsible for InlA – E-cadherin dependent entry of *L. monocytogenes* into trophoblasts (26). We have previously shown that RAPD type 9 strains do invade Caco-2 cells, which is another InlA – E-cadherin dependent cell line, to a lower level than strains of other origin and RAPD type (13) and we found in the present study that they were poor invaders of tropoblastic cells as well (Figure 1). We have identified a single point mutation in the four RAPD type 9 strains resulting in the formation of a premature stop codon (PMSC) that can explain the lower invasion ability of the RAPD type 9 strains. Whilst previous studies have demonstrated that a strain lacking *inlA* is attenuated in its ability to invade cells from the placental barrier (2, 9,26), our study is the first to demonstrate that strains with PMSC in *inlA* are affected in invasion of placental cells. The PMSC is at position 1473 which is at the same position as in French strains that were low invasive in Caco-2 cells (33). However, strains with truncated InlA cannot be regarded as non-virulent, as two large epidemiological studies found that a few cases of maternofetal listeriosis or bacteremia were caused by strains expressing truncated InlA (9,12), Another reason for low invasion into Caco-2 cells could be a low *inlA* transcript level, and some outbreaks have been caused by strains expressing low levels of *inlA* (38).

We also identified a small deletion of nine nucleotides in *inlA* at position 2214 in strain 4810-98, which is in the membrane anchoring region of InlA (30). As seen from the invasion data from both Caco-2 cells and JAR cells, this deletion does not influence the role of InlA in cell invasion, since strain 4810-98 invades both cell types to the same high level as strains with no deletions. This

small deletion is also seen in *L. monocytogenes* strain H7858, which is a serotype 4b frankfurter isolate from the multistate outbreak of 1998-1999 (6), however no phenotypic consequence of this small deletion has been described. The strain used in this study did all invade the L929 fibroblastic cells to the same level meaning that *inlA* is not involved in the invasion into this cell type.

As described, InlB is important for the interaction with several cell line receptors, however, full expression of InlB may not be a prerequisite for virulence as Nightingale et al (31) showed that the full genome sequenced strain F2365, which is an isolate from the 1985 listeriosis epidemic in California, contains a stop codon in *inlB* at position 100 and the strain will therefore not translate a full length InlB. We did, however, expect that InlB was important for invasion of *L. monocytogenes* into L929 fibroblast cells as it has been demonstrated that for instance single point mutations in InlB affects invasion into Vero cells (46). We found the same single point mutations at amino acid position 117 and 132 but these did not affect the fibroblast invasion. The SPM are located in the leucine-rich region of InlB, which is important for the interaction with the Met receptor (41). The contradiction between our results and the results seen by Temoin et al. (46) could be because of the studies used two different cell lines (Vero cells and L929 cells), or be due to the presence of other mutations in other genes.

When *L. monocytogenes* is spreading from one cell to the neighboring cell, ActA is responsible for the formation of the actin tail and strains lacking ActA are less virulent and unable to form plaques (5). ActA is also required for crossing of the fetoplacental barrier in both guinea pigs and mice (3,23). Surprisingly, the a*ctA* from several of the strains used in the present study had a deletion of 105 nucleotides (35 amino acids) in the central region of proline-rich repeats required for binding to vasodilator-stimulated phosphoprotein (VASP) (Figure 3B). This deletion has been described in several other *L. monocytogenes* strains (16,29,44,50) but the phenotype of the strains appears not to change. Jiang et al. (16) have showed that a strain containing the deletion is unable to form

plaques in the mouse fibroblastic cell line L929, but the control strain and the deletion strain in that study were not isogenic and therefore this could be due to other genetic differences. Accordingly, neither Solokovic et al. (44), Chen et al. (8) nor Morishii et al (29) found any correlation between plaque formation and *actA* deletion in either the rat epithelial cell line L2 or L929. Our results indicate that full length ActA is not obligatory for full invasion as the deletion of 105 nucleotides in *actA* did not affect cell to cell spread. The ability of a RAPD type 9 strain to spread to guinea pig fetuses cannot be caused by an increase capacity to cell-to-cell-spread as these strains showed average spread in terms of plaque size and numbers.

The paradox that an otherwise low virulent strain (La111) that harbors a mutation in *inlA* is able to infect guinea pig fetuses indicate that InlA does not play a crucial role in placental infection of guinea pigs. This is supported by another study in pregnant guinea pigs (2) as well as a study in pregnant mice (23). In contrast, full length *inlA* is important for human placental infection as demonstrated by human epidemiologic studies as well as studies with human trophoblastic cells and placental explants (2,9,12,26). The discrepancy between *in vivo* (guinea pig) and *in vitro* (cell lines) results is probably explained by the species-specificity of InlA and InlB. InlA interacts with the human and guinea pig E-cadherin but not with the mouse E-cadherin (24), whereas InlB interacts with the human and mouse Met receptor but not with the guinea pig Met receptor (20). Like humans, the gerbil is permissive to both the InlA – E-cadherin and the InlB – Met pathways and it has recently been established that InlA and InlB have interdependent roles in fetoplacental invasion in this species. Consequently, *L. monocytogenes* only targets the placenta *in vivo*, if both InlA and InlB pathways are functional (9).

Even though our results demonstrate the necessity of a full functional InlA, strains with PMSC in *inlA* can, albeit rarely, cause human listeriosis (9,12), suggesting that other pathways can be involved in the infection and/or that other factors specific to either the host or the strain of *L. monocytogenes* influence the course of infection. This could explain why the RAPD type 9 strain

with a PMSC in *inlA* can infect guinea pig fetuses as other pathways may be important in this species. In addition, other strain-specific factors, like the ability to survive and grow in the food matrix within which it is delivered to the host, can also influence the infectious potential as also speculated by Roberts et al (38).

The key question in this study was if the otherwise low-virulent RAPD type 9 strains had a preference for the placenta and thus would pose a particular risk to pregnant women. It is striking that the four tested RAPD type 9 strains all had the exact same sequence (and mutations) for all three sequenced virulence genes when compared to the EGD-e genome indicating that these strains are genetically highly similar. They have been isolated from different processing environments over a period of 12 years and have in all environments been identified as persistent strains (51). We have shown that the RAPD type 9 strains did not invade human placental trophoblasts more efficiently than other strains, probably due to the presence of PMSC in their *inlA*. In addition, they did not show enhanced intracellular replication in these trophoblasts nor increased ability to spread from cell to cell in mouse fibroblasts. Based on these results and in accordance with Jensen et al. (14) and Disson et al (9), we conclude that the RAPD type 9 strains can still be regarded as low-virulent with respect to human listeriosis and do not pose an increased risk to pregnant women in particular.

Acknowledgement

We thank Vi Nguyen for the excellent handling of the cell lines. AH was supported by “Microbial Opportunistic Pathogens” (grant #2052-03-0013) and by The Danish Research Council for Technology and Production Sciences (project 274-08-042) and CTG was funded by a Ph.D. grant from the Technical University of Denmark.

Table 1: Origin, serotype, lineage, RAPD type, cell invasion and virulence gene sequence of the *Listeria monocytogenes* strains used in the present study. The maternofetal strains are described according to gestation day and the out come of the fetus (L: Live, D: Dead). Presence of mutations in the virulence genes, *inlA*, *inlB* and *actA* is indicated. PMSC: Premature stop codon, FL: Full-length, Del: Deletion, SPM[1,2]: Single point mutation [1](alanine to threonine) or [2](valine to isoleucine), NM: No mutation. Invasion levels are presented as cfu/ml.

Strain	Origin	Serotype	Lineage	RAPD type	*inlA*	*inlB*	*actA*	Invasion Caco-2	Invasion L929	Invasion JAR	No of Plaque pr. 10^2 invaded bacteria in L929 cells	Reference
N53-1	Smoke house equipment	1/2a	2	9	PMSC	SPM[1,2]	Del	$10^{2,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^4	72	(51)
La111	Smoke house equipment	1/2a	2	9	PMSC	SPM[1,2]	Del	$10^{2,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^4	75	(49)
H13-1	Smoke house equipment	1/2a	2	9	PMSC	SPM[1,2]	Del	$10^{2,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^4	81	(51)
M103-1	Slaughter house equipment	1/2a	2	9	PMSC	SPM[1,2]	Del	$10^{2,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^4	140	(51)
La22	Cold smoked salmon	1/2a	2	12	FL	SPM[1,2]	FL	$10^{5,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^5-10^6	104	(48)
7418	Spreadable sausage	1/2b	1	14	FL	SPM[2]	FL	10^5-$10^{6,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^6	54	(21)
3849-97	Maternofetal (35+6,L)	4	1	nt[a]	FL	SPM[2]	Del	10^5-10^6	$5{\cdot}10^1$-$1{\cdot}10^2$	10^5	85	(42)
3272-03	Maternofetal (35+2, L)	1	2	nt	FL	SPM[1,2]	FL	10^5-10^6	$5{\cdot}10^1$-$1{\cdot}10^2$	10^5-10^6	292	(42)
3495-04	Maternofetal (31+4, L)	1	1	nt	FL	NM	Del	10^5-10^6	$5{\cdot}10^1$-$1{\cdot}10^2$	10^5-10^6	101	(42)
4810-98	Maternofetal (38+4, D)	4	1	nt	Del	SPM[2]	FL	10^5-10^6	$5{\cdot}10^1$-$1{\cdot}10^2$	10^6	63	(42)
12443	Monkey, maternofetal	1/2a	2	73	FL	SPM[1,2]	Del	10^5-$10^{6,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^6	133	(43)
Scott A	Human, clinical	4b	1	72	FL	SPM[2]	FL	$10^{6,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^6	234	[c]
4446	Human, clinical	4b	1	71	FL	SPM[2]	Del	10^5-$10^{6,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^5-10^6	51	(21)
LO28	Human, fecal	1/2c	2	69	PMSC	NM	FL	$10^{3,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^4-10^5	84	(47)
EGD	Rabbit, 1926	1/2a	2	68	FL	NM	FL	$10^{4,b}$	$5{\cdot}10^1$-$1{\cdot}10^2$	10^4-10^5	87	[d]

[a] The bacteria did not belong to any of the RAPD types represented by the other strains but a specific RAPD type was not assigned
[b] The results have previously been published in Jensen et al. (13) and Jensen et al. (15) and unpublished results.
[c] The strain was kindly provided by Campden Food and Drink Association, UK.
[d] The strain was kindly provided by Werner Goebel, University of Würzburg, Germany.

Table 2: Primers used for sequencing of *Listeria monocytogenes* virulence genes.

Primer	Sequence (5' – 3')	Annealing temperature	Reference
1F	GGA AAA ATG TGC TGG AAC	45°C	This study
1R	CGG GTC TAT ATC CGT TAT CTG A		This study
1.5F	ATC GAT GGA GTG GAA TAC TT	48°C	This study
1.5 R	GTG CCT ATA TCT TTT AAC TGG TTA C		This study
2F	CCG CTA GCT AAT TTG ACG A	41.7°C	This study
2R	GCA AGT GAG CTT ACG TCA		This study
4F	CTT GGA GCT AAC CAA ATA AGT AAC A	48°C	This study
4R	TTT TAC GGG CTT AGC TGG TT		This study
4.5 F	GTG AAA AAT GTG ACT GGC GC	58°C	This study
4.5 R	TCC GTC GCA AAA TCC CAT TT		This study
InlA seq F	GTG GAC GGC AAA GAA ACA AC	58°C	(30)
InlA R	ATA TAG TCC GAA AAC CAC ATC T		(30)
actA F	AGT CAG TTG CGG ATG CTT CTG	58°C	This study
actA R	TCT GTT GTC TCG CTG TTT TCG		This study
InlB1-2F	GAT GTG GTT TTC GGA CTA TAT	47°C	This study
InlB1-2R	GCT AAT GCT CTT AAA TCG CT		This study
InlB2 F	TTC TTT GGA GCA TAA TGG TA	48°C	This study
InlB2 R	CGC TTT AGT CCA GCC AAT AT		This study
InlB3F	TAC TGA AAA AAA TGG TGG GCA T	47°C	This study
InlB3R	TTG ACC TTC GAT GGT TGC TT		This study

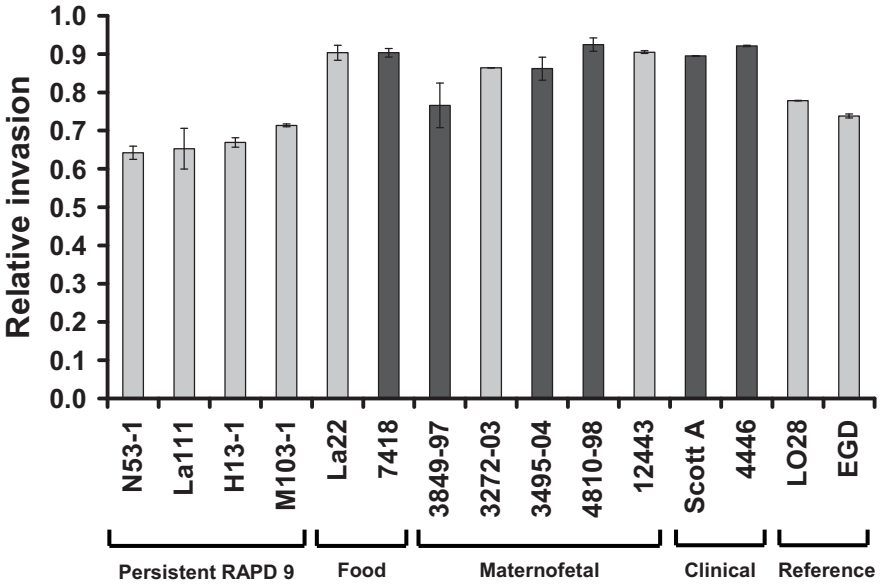
Legends

Figure 1: Invasion of *L. monocytogenes* strains into human trophoblastic JAR cells. Strains were grown in BHI broth at 37°C for 20 hours and adjusted to 1.0×10^6 CFU/ml before infection of the JAR monolayer. Invasion is expressed as the number of intracellular CFU/ml relative to the number of CFU/ml added to the well. Strains have been sorted according to origin. Dark columns represent lineage I strains and light columns represent lineage 2 strains. Columns are average from one trial carried out in duplicate and error bars are standard deviations. The results are representative of three independent experiments.

Figure 2: Plaque formation by *L. monocytogenes* in mouse fibroblastic L929 cells. Strains were grown in BHI broth at 37°C for 20 hours and adjusted to 5×10^4 CFU/ml before infection of the L929 monolayer. Data is expressed as the number of plaques for 10^2 invaded bacteria. Strains have been sorted according to their origin. Dark columns represent lineage I strains and light columns represent lineage 2 strains. Check patterned columns represent strains with a 105 bp deletion in *actA.* Columns are average from one trial carried out in duplicate and error bars are standard deviations. The results are representative of three independent experiments.

Figure 3: Amino acid sequence of sequence of InlA (A), InlB (B) and ActA (C) from *Listeria monocytogenes* strains. The InlA from the four RAPD type 9 strains has a premature stop codon (PMSC) at position 492 leading to a truncated InlA. The InlB from the four RAPD type 9 strains has several single point mutations. The ActA has, for several of the strains, a deletion of 35 amino acids in the vasodilator-stimulated phosphoprotein region.

Figure 1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0.6
0.5
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0.3
0.2
0.1
0.0
N53-1
La111
H13-1
M103-1
La22
7418
3849-97
3272-03
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12443
Scott A
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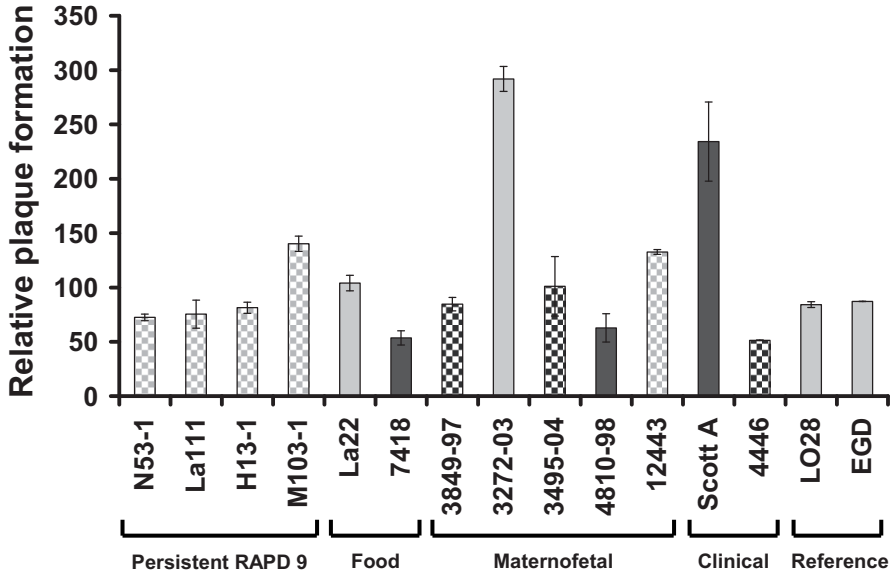
LO28
EGD
Persistent RAPD 9
Food
Maternofetal
Clinical
Reference

Figure 2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250
200
150
100
50
0
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La111
H13-1
M103-1
La22
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3849-97
3272-03
3495-04
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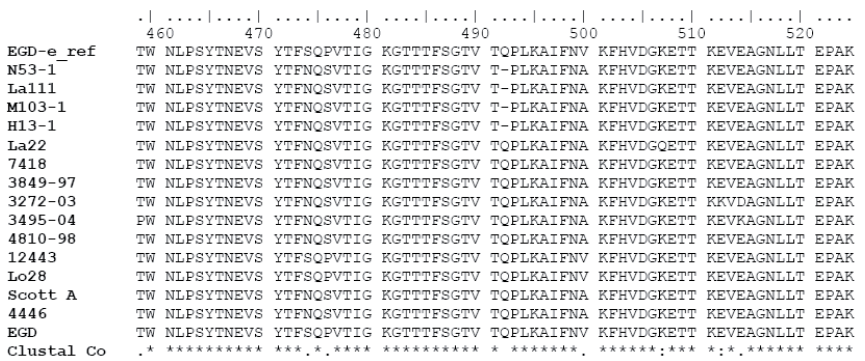
12443
Scott A
4446
LO28
EGD
Persistent RAPD 9
Food
Maternofetal
Clinical
Reference

Figure 3

A



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M103-1          TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV T-PLKAIFNA KFHVDGKETT KEVEAGNLLT EPAK
H13-1           TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV T-PLKAIFNA KFHVDGKETT KEVEAGNLLT EPAK
La22            TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV TQPLKAIFNA KFHVDGQETT KEVEAGNLLT EPAK
7418            TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV TQPLKAIFNA KFHVDGKETT KEVEAGNLLT EPAK
3849-97         TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV TQPLKAIFNA KFHVDGKETT KEVEAGNLLT EPAK
3272-03         TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV TQPLKAIFNA KFHVDGKETT KKVDAGNLLT EPAK
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4810-98         TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV TQPLKAIFNA KFHVDGKETT KEVEAGNLLT EPAK
12443           TW NLPSYTNEVS YTFSQPVTIG KGTTTFSGTV TQPLKAIFNV KFHVDGKETT KEVEAGNLLT EPAK
Lo28            TW NLPSYTNEVS YTFSQPVTIG KGTTTFSGTV TQPLKAIFNV KFHVDGKETT KEVEAGNLLT EPAK
Scott A         TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV TQPLKAIFNA KFHVDGKETT KEVEAGNLLT EPAK
4446            TW NLPSYTNEVS YTFNQSVTIG KGTTTFSGTV TQPLKAIFNA KFHVDGKETT KEVEAGNLLT EPAK
EGD             TW NLPSYTNEVS YTFSQPVTIG KGTTTFSGTV TQPLKAIFNV KFHVDGKETT KEVEAGNLLT EPAK
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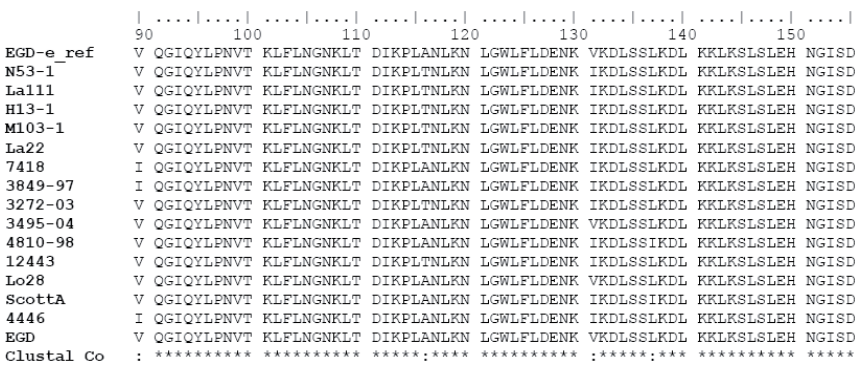

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Lo28            V QGIQYLPNVT KLFLNGNKLT DIKPLANLKN LGWLFLDENK VKDLSSLKDL KKLKSLSLEH NGISD
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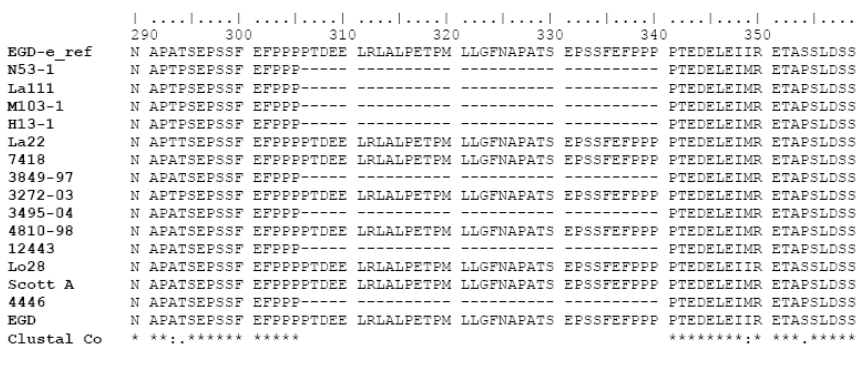

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N53-1           N APTPSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSS
La111           N APTPSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSS
M103-1          N APTPSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSS
H13-1           N APTPSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSS
La22            N APTTSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSS
7418            N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSS
3849-97         N APATSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSS
3272-03         N APTPSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSS
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4810-98         N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSS
12443           N APATSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSS
Lo28            N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIIR ETASSLDSS
Scott A         N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIMR ETAPSLDSS
4446            N APATSEPSSF EFPPP----- ---------- ---------- ---------- PTEDELEIMR ETAPSLDSS
EGD             N APATSEPSSF EFPPPPTDEE LRLALPETPM LLGFNAPATS EPSSFEFPPP PTEDELEIIR ETASSLDSS
Clustal Co      * **:.****** *****                                        ********:* ***.*****
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