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ROLE OF WALL TEICHOIC ACID L-RHAMNOSYLATION IN *LISTERIA MONOCYTOGENES* RESISTANCE TO ANTIMICROBIAL PEPTIDES AND SURFACE PROTEIN ANCHORING

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According to the relevant national legislation, the author clarifies that this thesis includes data from the publications listed below, and declares that he participated actively in the conception and execution of the experiments that produced such data, as well as in their interpretation, discussion and writing.

PUBLICAÇÕES / PUBLICATIONS

- **Carvalho F**, Atilano ML, Pombinho R, Covas G, Gallo R, Filipe SR, Sousa S, Cabanes D (2015) L-rhamnosylation of *Listeria monocytogenes* wall teichoic acids promotes resistance to antimicrobial peptides by delaying interaction with the membrane. *PLoS Pathog* **11**(5):e1004919

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ABSTRACT

Listeria monocytogenes is an opportunistic Gram-positive pathogen and the cause of human listeriosis, a severe and often fatal foodborne disease that targets immunocompromised hosts. This pathogenicity results from the action of numerous virulence proteins, many of which are associated with the cell envelope. The cell wall of *L. monocytogenes* is densely decorated with wall teichoic acids (WTAs), a class of anionic glycopolymers known to play key roles in bacterial physiology, such as protection against antimicrobial peptides (AMPs) and control of autolysin activity. In other Gram-positive bacteria, WTA modification by amine-containing groups such as D-alanine was largely correlated with increased resistance to AMPs and shown to influence autolytic levels. However, in *L. monocytogenes,* where WTA modification is achieved solely by glycosylation, WTA-dependent mechanisms of AMP resistance and autolytic regulation remain unknown.

In this work, we show that the *L. monocytogenes* WTA L-rhamnosylation requires the *rmlACBD* locus, which encodes the biosynthetic pathway for L-rhamnose, and the upstream-flanking gene *rmlT*, encoding a putative rhamnosyltransferase. We then demonstrate for the first time that this particular WTA tailoring mechanism promotes AMP resistance, sustains physiological levels of bacterial autolysis and supports virulence mechanisms. In particular, we show that L-rhamnosylated WTAs delay the crossing of the *L. monocytogenes* cell wall by AMPs and postpone their contact with the plasma membrane, through a decrease of the cell wall permeability to AMPs. Importantly, we reveal the contribution of this WTA decoration for *L. monocytogenes* survival and virulence in a mouse model of infection. In addition, we implicate L-rhamnosylated WTAs in the maintenance of optimal levels of autolytic activity and host cell invasion, through a previously unknown contribution to an efficient surface anchoring of representative members of the *L. monocytogenes* GW protein family.

Altogether, these results demonstrate that WTA glycosylation mechanisms are also important for a variety of biological processes linked with bacterial physiology and pathogenesis.

RESUMO

Listeria monocytogenes é uma bactéria Gram-positiva patogénica causadora da listeriose humana. Esta doença afecta sobretudo hospedeiros imunocomprometidos, onde pode evoluir até se tornar fatal. A patogenicidade de *L. monocytogenes* resulta da acção de inúmeros factores de virulência, muitos dos quais estão associados com o invólucro bacteriano. A parede celular desta bactéria é densamente decorada com ácidos teicoicos (ATs), uma família de glicopolímeros aniónicos conhecidos pelos seus variados papéis na biologia bacteriana, como por exemplo protecção contra péptidos antimicrobianos (PAMs) e controlo da actividade autolítica. Noutras bactérias Gram-positivas, a modificação dos ATs com grupos aminados (p.e. D-alanina) está intimamente relacionada com resistência à actividade de PAMs e influencia os níveis de autólise. No entanto, em *L. monocytogenes* – cujos ATs são apenas modificados com açúcares – os mecanismos de resistência a PAMs e de regulação da actividade autolítica dependentes de ATs permanecem desconhecidos.

Neste trabalho, mostramos que a L-ramnosilação dos ATs de *L. monocytogenes* precisa dos genes *rmlACBD*, que codificam a via biosintética da L-ramnose, e do gene *rmlT*, que codifica para uma potencial ramnosiltransferase. Demonstramos pela primeira vez que este mecanismo particular de substituição de ATs promove a resistência a PAMs, sustenta níveis fisiológicos de autólise, e apoia mecanismos de virulência. Mostramos especificamente que os ATs ramnosilados atrasam a travessia da parede celular de *L. monocytogenes* pelos PAMs e adiam o contacto destes com a membrana, através de uma diminuição da permeabilidade da parede celular a estes péptidos. Revelamos também a importante contribuição desta decoração de ATs para a sobrevivência e virulência de *L. monocytogenes in vivo*, usando murganhos como modelo de infecção. Ainda, responsabilizamos os ATs ramnosilados pela manutenção de níveis óptimos de actividade autolítica e invasão celular, através da contribuição previamente desconhecida para a eficiente ancoragem à superfície de *L. monocytogenes* de membros representativos da família de proteínas com domínios GW.

No seu conjunto, estes resultados demonstram que os mecanismo de glicosilação de ATs são igualmente importantes para uma variedade de processos biológicos associados com a fisiologia e patogénese bacteriana.

LIST OF ABBREVIATIONS

aa – amino acid

ABC – ATP-binding cassette

Ala – alanine

AMP – antimicrobial peptide

Arg - arginine

Arp2/3 – actin-related proteins 2 and 3

BHI – brain and heart infusion

BMAP – bovine antimicrobial peptide

BSA – bovine serum albumin

C55-P – undecaprenyl-phosphate

C-terminal – carboxy-terminal

CAMP – cationic antimicrobial peptide

CAP-18 – cationic antimicrobial protein of 18 kDa

CDC – cholesterol-dependent cytolysin

cDNA – complementary DNA

CDP – cytidine diphosphate

CNS – central nervous system

CFTR – cystic fibrosis transmembrane receptor

CFU – colony-forming unit

CRAMP – cathelicidin-related antimicrobial peptide

D – dextrorotary

Da – dalton

DAG – diacylglycerol

$DiOC_2(3)$ – 3,3'-diethyloxacarbocyanine

DNA – deoxyribonucleic acid

DNase – deoxyribonuclease

dTDP – deoxythymidine diphosphate

E-cad – E-cadherin or epithelial cadherin

EDTA – ethylenediamine tetracetic acid

5-FAM – 5-carboxyfluorescein

FSC – forward scatter

GAG – glycosaminoglycan

Gal – galactose

gC1qR – receptor for the globular component of complement C1q

GILT - gamma-interferon-inducible lysosomal thiol reductase

Glc – glucose

Glc*N*Ac – *N*-acetylglucosamine

Glu – glutamate

Gly – glycine

GroP – glycerol-phosphate

GT-A – glycosyltransferase fold A

GW – glycine-tryptophan dipeptide

HBD – human beta-defensin

HD – human defensin

HIV – human immunodeficiency virus

HMW – high-molecular weight

HNP – human neutrophil peptide

HPAEC-PAD – high-performance anion exchange chromatography coupled to pulsed amperometric detection

HPLC – high-performance liquid chromatography

HRP – horseradish peroxidase

IM – inner membrane

Inl – internalin

IR – inter-repeat

kDa – kilodalton

KO – knockout

L – levorotary

LAB – lactic acid bacteria

LB – lysogeny broth

LCP – LytR-CpsA-Psr protein

LLO – listeriolysin O

Lm – *Listeria monocytogenes*

LMW – low-molecular weight

LPS - lipopolysaccharide

LRR – leucine-rich repeat

LTA – lipoteichoic acid

LysM – lysin motif

Man*N*Ac – *N*-acetylmannosamine

Man-PTS – mannose-specific phosphotransferase system

MAPK – mitogen-activated protein kinase

***m*Dpm** – *meso*-2,6-diaminopimelic acid

MES - 2-(*N*-morpholino)ethanesulfonic acid

MFI – mean fluorescence intensity

MOPS – 3-(*N*-morpholino)propanesulfonic acid

mRNA – messenger RNA

Mur*N*Ac – *N*-acetylmuramic acid

N-terminal – amino-terminal

NAGase – *N*-acetylglucosaminidase

NAMase – *N*-acetylmuramidase

NF-κB – nuclear factor kappa B

NK – natural killer

OM – outer membrane

PAGE – polyacrylamide gel electrophoresis

PBP – penicillin-binding protein

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PC-PLC – phosphatidylcholine-specific phospholipase C

PE – phosphatidylethanolamine

PEST – proline-glutamate-serine-threonine tetrapeptide

PG – phosphatidylglycerol

PI-PLC – phosphatidylinositol-specific phospholipase C

Pro - proline

PRR – proline-rich repeat

PTM – post-translational modification

qPCR – quantitative real-time PCR

RboP – ribitol-phosphate

RNA – ribonucleic acid

RNase – ribonuclease

ROS – reactive oxygen species

rpm – rotations per minute

rRNA – ribosomal RNA

RTD – rhesus macaque theta-defensin

SDS – sodium dodecylsulfate

SH3 – Src homology 3

SIC – streptococcal inhibitor of complement

SSC – side scatter

SUMO – small ubiquitin-like modifier protein

TA – teichoic acid

TCA – trichloroacetic acid

TLR – Toll-like receptor

tRNA – transfer RNA

Trp - tryptophan

UDP – uridine diphosphate

VASP – vasodilator-stimulated phosphoprotein

WASP – Wiskott-Aldrich syndrome protein

WT – wild type

WTA – wall teichoic acid

CHAPTER I

INTRODUCTION

A. *LISTERIA MONOCYTOGENES*

A.1. History

Our knowledge about *Listeria monocytogenes* (*Lm*) goes back as far as 1926, when the identification of this bacterium was first reported by Murray and colleagues, in the aftermath of an epidemic outbreak among rabbits and guinea pigs in their laboratory in Cambridge, England. They named the new species *Bacterium monocytogenes* due to the increased number of monocytes observed in the blood of animals infected with sub-lethal doses of this microorganism (Murray *et al.* 1926). The following year, Pirie unknowingly reported the isolation of the same species in South Africa, which he named *Listerella hepatolytica*, in honor of Lord Joseph Lister, the father of antiseptic surgery (Pirie 1927). Acknowledging Murray's discovery, Pirie changed the species name to *Listerella monocytogenes*, but confronted with the prior use of *Listerella* for a genus of slime molds, he proposed its renaming in 1940 to the current form (Pirie 1940).

Although human cases had already been reported (Nyfeldt 1929, Reiss *et al.* 1951), they were highly sporadic and *Lm* infection was essentially regarded as a zoonosis. It was only in 1981 that *Lm* was recognized as a human food-borne pathogen, after a severe listeriosis outbreak in Canada related with consumption of contaminated food resulted in an elevated percentage of case deaths (Schlech *et al.* 1983). Further food-related outbreaks during the following two decades consolidated the status of *Lm* as a microorganism of public health concern (Swaminathan and Gerner-Smidt 2007a).

A.2. Taxonomy, phylogeny and classification

Listeria is one of two genera – the other is *Brochothrix* – of the Listeriaceae family, which in turn belongs to the order Bacillales, class Bacilli, and phylum Firmicutes of the domain Bacteria. Other genera closely related to *Listeria* include *Bacillus* and *Staphylococcus*. Since its discovery, *Lm* was for a long time the only species within its genus. However, in the second half of the 20th century, with the aid of biochemical and genetic typing tools, Seeliger and Rocourt were able to

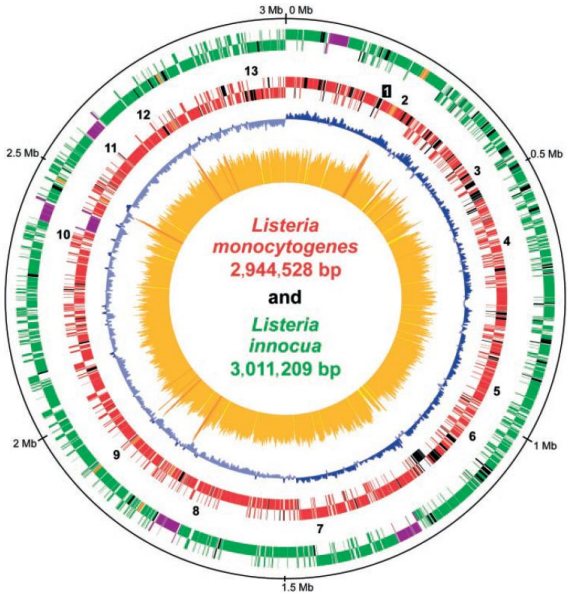

Fig. 1. Circular genome maps of *Lm* EGD-e and *L. innocua* CLIP 11262, showing the position and orientation of genes. From the outside: Circles 1 and 2, *L. innocua* and *Lm* genes on the plus and minus strands, respectively. Color code: green, *L. innocua* genes; red, *Lm* genes; black, genes specific for *Lm* or *L. innocua*, respectively; orange, rRNA operons; purple, prophages. Numbers on the second circle indicate the position of known virulence genes: 1, virulence locus (*prfA-plcA-hly-mpl-actA-plcB*); 2, *clpC*; 3, *inlAB;* 4, *iap*; 5, *dal*; 6, *clpE*; 7, *lisRK*; 8, *dat*; 9, *inlC*; 10, *arpJ*; 11, *clpP*; 12, *ami*; 13, *bvrABC*. Circle 3, G/C bias (G+C/G-C) of *Lm*. Circle 4, G+C content of *Lm* (<32.5% G+C in light yellow, 32.5 to 43.5% in yellow, and >43.5% G+C in dark yellow). The scale in megabases (Mb) is indicated on the outside of the genome circles, with the origin of replication at position 0. (From Glaser *et al.* 2001)

distinguish and identify five novel species: *L. innocua* (Seeliger 1981), *L. welshimeri*, *L. seeligeri* (Rocourt and Grimont 1983), *L. ivanovii* (formerly *L. bulgarica*) (Seeliger *et al.* 1984) and *L. grayi* (Larsen and Seeliger 1966, Rocourt *et al.* 1992). Recently, the *Listeria* genus has undergone a major expansion, from six to seventeen species, with the identification of *L. marthii* and *L. rocourtiae* (Graves *et al.* 2010, Leclercq *et al.* 2010); *L. fleischmannii* and *L. weihenstephanensis* (Bertsch *et al.* 2013, Halter *et al.* 2013); and *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia* and *L. grandensis* (den Bakker *et al.* 2014). Among these, *Lm* (infects humans and animals) and *L. ivanovii* (infects mainly livestock) are the only confirmed pathogenic species; the remaining live as apathogenic saprophytes in nature (Rocourt and Grimont 1983, Graves *et al.* 2010, Leclercq *et al.* 2010, Bertsch *et al.* 2013, Halter *et al.* 2013, den Bakker *et al.* 2014).

The turn of the century introduced post-genomics to the *Listeria* research field, after the complete genome sequences of *Lm* (EGD-e) and *L. innocua* (CLIP 11262) (Fig. 1) were published (Glaser *et al.* 2001). Soon after, whole-genome sequences of other *Lm* strains also became available (Nelson *et al.* 2004, Kuenne

et al. 2013) and, progressively, those of other *Listeria* species (Hain *et al.* 2006b, Steinweg *et al.* 2010, Buchrieser *et al.* 2011). Comparative genomics enabled – among other possibilities – the identification of differences important for the comprehension of the phylogenetic relationship of *Listeria* spp. To understand the evolution of *Listeria* pathogenicity, Schmid and colleagues made a phylogenetic analysis focused on the comparison of multiple virulence-associated loci in the different species. Their analyses indicated that *L. grayi* was likely the first to diverge within the genus and lose its pathogenic capacity, followed by the branching of *Lm* and *L. innocua* into one lineage, and of *L. ivanovii*, *L. seeligeri* and *L. welshimeri* into another (Schmid *et al.* 2005).

Early on, *Listeria* classification relied on the serotyping of somatic (O) and flagellar (H) antigens. Based on this method, 16 *Listeria* serotypes were identified, 13 of which are found in *Lm* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) (Seeliger and Höhne 1979, Seeliger and Langer 1989, Gorski 2008). Serotyping, however, is not very specific due to the high antigenic overlap between serotypes. Thus, more specific genetic-based typing methodologies (genotyping) led to the organization of *Lm* serotypes into four lineages: lineage I (1/2b, 3b, 4b, 4d and 4e), lineage II (1/2a, 1/2c, 3a and 3c), lineage III (4a and 4c), and lineage IV (7) (Orsi *et al.* 2011).

A.3. General features

Lm is a small, rod-shaped (0.5×1–2 µm), non-encapsulated, non-sporulating, facultative anaerobic, Gram-positive bacterium (Rocourt and Buchrieser 2007). It expresses four-to-six peritrichous flagella at temperatures up to 25 °C, which confer motility to *Lm* while in the environment. This flagellar motility decreases with further increase in temperature until it is completely lost at 37 °C (temperature inside a host) due to transcriptional repression of the flagellar assembly system (Peel *et al.* 1988, Gründling *et al.* 2004).

Lm is a physiologically robust bacterium, able to grow under a broad range of temperatures (<0 to 45 °C) and pH (4.3 to 9, optimal at 7), and high osmotic pressures (up to 10% NaCl) (Shahamat *et al.* 1980, Junttila *et al.* 1988, Parish and Higgins 1989, George and Lund 1992). These properties make *Lm* a virtually

Table 1. General features of published *Listeria* genome sequences.[a,b]

	L. monocytogenes EGD-e	*L. monocytogenes* F2365	*L. innocua* CLIP11262	*L. welshimeri* CIP8149
Chromosome size (bp)	2,944,528	2,905,310	3,011,209	2,814,130
% G+C content	38	38	37.4	36.4
ORFs	2,853	2,847	2,973	2,780
% Coding ORFs	89.2	88.4	89.1	88.7
Prophages	1	2	5	1
Plasmids	–	–	1 (79 ORFs)	–
Strain-specific genes	61	51	78	208
Transposons	1 (Tn916-like)	–	–	–
rRNA genes	6	6	6	6
tRNA genes	67	67	66	66

a) Adapted from Buchrieser 2007.
b) bp, base pairs; G+C, guanine and cytosine; ORF, open reading frame.

ubiquitous microorganism, able to adapt to the demands of a wide variety of ecological environments. Indeed, *Lm* has been isolated from soil, water, sewage, plants and animal feces (Fenlon 1999), where it lives as a saprophyte (Weis and Seeliger 1975).

Despite the elevated phenotypic similarity within the genus, *Lm* can be distinguished from other *Listeria* spp. through a set of biochemical tests that assess hemolytic (red blood cell-lysing) activity and acid production (fermentation) from carbohydrate sources (Rocourt *et al.* 1983). In the case of *Lm*, it is the only hemolytic *Listeria* that ferments L-rhamnose but not D-xylose.

Since the publication of the first *Listeria* genomes in 2001 (Glaser *et al.* 2001), multiple other species and strains have also had their genomes sequenced and analyzed. With few exceptions, the average *Lm* genome size varies between 2.7 and 3.0 Mb – with an average G+C content of about 38%, typical of Firmicutes – and contains about 2900 protein-coding sequences (Table 1) (Hain *et al.* 2006a, Buchrieser 2007). These numbers are very similar to those of other *Listeria* spp., such as *L. innocua* or *L. welshimeri* (Buchrieser 2007). Indeed, all *Listeria* genomes show a highly conserved organization, which reflects the strong phylogenetic closeness between listeriae (Buchrieser *et al.* 2003). Nonetheless, they also display genomic differences that are likely to be associated with inter- or intra-specific variations of certain phenotypic parameters, such as pathogenicity. In fact, a critical genetic difference between *Lm* and its non-pathogenic relatives *L. innocua* and *L. welshimeri* concerns the most important *Lm* virulence genes,

which are all absent from the homologous regions in both avirulent species (Schmid *et al.* 2005, Buchrieser 2007).

A.4. Listeriosis

A.4.1. Epidemiology

The transmission of *Lm* to humans is achieved mainly through the consumption of contaminated food, although there were reports of infection transmitted between humans or acquired from animals (Allerberger and Wagner 2010). Due to its remarkable fitness, *Lm* can survive to most standard industrial food-preserving methods (e.g. refrigeration, acid- or salt-based treatments) to persist and grow in a variety of raw and processed foods, including meats (e.g. charcuterie and deli), seafood, produce (fruits and vegetables), unpasteurized milk and dairy products (e.g. soft cheeses) (Swaminathan and Gerner-Smidt 2007b). Despite the environmental widespreadness of *Lm* and the continuous exposure of humans to this pathogen, listeriosis has a very low incidence in humans, with 1–10 cases per million people reported every year. In recent years, the total number of annual cases has been increasing, particularly in developed countries (Denny and McLauchlin 2008, Goulet *et al.* 2008). In contrast to its low occurrence, the average rate of clinical case deaths reaches 20–30%, making it one of the most deadly food-borne infections, only surpassed by salmonellosis (Gould *et al.* 2013). Over 95% of all reported human listeriosis cases have been caused by *Lm* strains belonging to serotypes 1/2a, 1/2b, 1/2c, and 4b. Serotype 4b accounts for the majority of epidemic outbreaks, while serogroup 1/2 has been mostly associated with sporadic cases (Jacquet *et al.* 2002, Goulet *et al.* 2006).

A.4.2. Pathophysiology

Following its ingestion, *Lm* must be able to survive through the aggressive environment of the gastric compartment before arriving at the intestinal lumen. Once there, bacteria can penetrate further into the host organism by crossing the intestinal epithelium (Fig. 2). Depending on the host species, this trans-intestinal

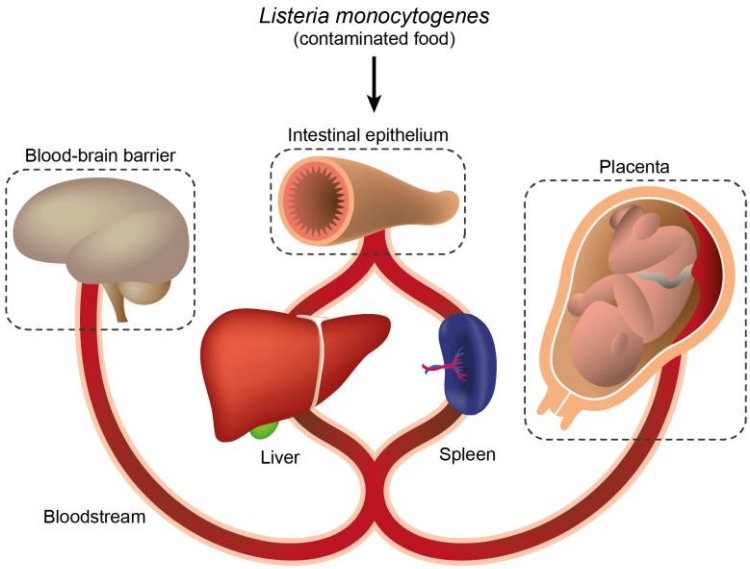

Fig. 2. Schematic representation of the successive steps of human listeriosis.

passage occurs mainly via enterocytes (humans, gerbils and rabbits) or M-cells in Peyer's patches (murines) (Vazquez-Boland *et al.* 2001, Lecuit 2005, Lecuit 2007). After translocation, bacteria are carried in the lymph and the blood to the spleen and the liver, the major target organs for *Lm* colonization, where they are quickly taken up by resident macrophages, such as Kupffer cells. In the liver, the majority of the captured bacteria are destroyed inside these phagocytic cells, but a substantial number is able to survive and infect nearby hepatocytes, where the *Lm* population can recover and spread to adjacent cells and tissues. If the hepatic infection is not contained by the host immune system, uncontrolled bacterial multiplication will lead to the freeing of *Lm* into the bloodstream, resulting in bacteremia (Vazquez-Boland *et al.* 2001, Zenewicz and Shen 2007). Blood-borne *Lm* can then migrate to and infect secondary target organs, such as the brain and placenta (with consequent infection of the fetus), by crossing both the blood-brain and the placental barriers (Fig. 2) (Vazquez-Boland *et al.* 2001, Lecuit 2005, Lamont *et al.* 2011, Disson and Lecuit 2012).

A.4.3. Clinical manifestations and treatment

The prime mechanism of host defense against *Lm* infection is cell-mediated immunity (Mackaness 1960, Zenewicz and Shen 2007). Thus, the clinical severity

of listeriosis is dependent on the functional status of the host immune system. In healthy immunocompetent hosts, listeriosis can be asymptomatic or, in the worst-case scenario, manifest as a self-limiting and short-term febrile gastroenteritis. Immunodepressed individuals, such as the elderly, pregnant women, neonates, HIV carriers, and those undergoing immunosuppressive treatments, cannot mount a proper T cell-mediated immune response against bacterial pathogens, and are thus much more susceptible to *Lm* infection (Vazquez-Boland *et al.* 2001, Swaminathan and Gerner-Smidt 2007b, Hernandez-Milian and Payeras-Cifre 2014). In these risk groups, listeriosis takes on a clinically more invasive and potentially lethal form, typically characterized by bacteremia, which can then evolve to systemic (septicemia) or more localized infections, either in the central nervous system (CNS) or in the fetoplacental system.

CNS infections are the most predominant form of invasive listeriosis in non-pregnant human adults (55–70% case reports), due to the tropism of *Lm* for nervous tissue (Vazquez-Boland *et al.* 2001, Hernandez-Milian and Payeras-Cifre 2014), and manifest primarily as meningitis but also as meningoencephalitis (Disson and Lecuit 2012). Maternofetal and early-onset neonatal listeriosis are the most common pregnancy-associated variants of the disease. They are elicited *in utero* – mainly during the third trimester, when the maternal immune system is weakened – with the placental translocation of *Lm* from the maternal blood to the fetus. Whereas the mother is hardly affected, displaying flu-like symptoms in the worst case, infection of the fetus can become systemic and result in abortion or pre-term delivery of a stillborn or a live but severely affected infant. Less frequent, late-onset neonatal listeriosis develops in week-old neonates, probably after having contacted with contaminated maternal fluids during delivery. Commonly associated symptoms include fever and meningitis, but also gastroenteritis and pneumonia (Vazquez-Boland *et al.* 2001, Lamont *et al.* 2011).

Antimicrobial therapy is the current standard treatment for listeriosis. It involves the intravenous administration of beta-lactamic antibiotics (ampicillin or penicillin) in combination with an aminoglycoside (e.g. gentamicin). Patients allergic to beta-lactams can be treated with alternative antimicrobial compounds, which include trimethoprim/sulfamethoxazole, erythromycin, vancomycin or fluoroquinolones. Pregnant women should not be given gentamicin, due to

potential teratogenic effects on the fetus. Treatment duration is variable but should typically last more than two weeks (Allerberger and Wagner 2010).

A.5. Cellular infection cycle

The remarkable capacity of *Lm* to overcome tight physiological barriers such as the intestinal epithelium, the placenta, and the blood-brain barrier (Lecuit 2005) comes from its ability to survive inside professional phagocytes and, more importantly, to invade non-phagocytic cells (e.g. epithelial and endothelial cells, fibroblasts and hepatocytes) (Cossart and Toledo-Arana 2008). Once inside a target cell, *Lm* proliferates and propagates the infection by spreading to other cells (Fig. 3).

When *Lm* first encounters a non-phagocytic host cell, it makes use of a set of surface proteins that enable its direct contact and stable adhesion to the cell membrane (adhesins). Almost concurrently, *Lm* induces its own internalization by

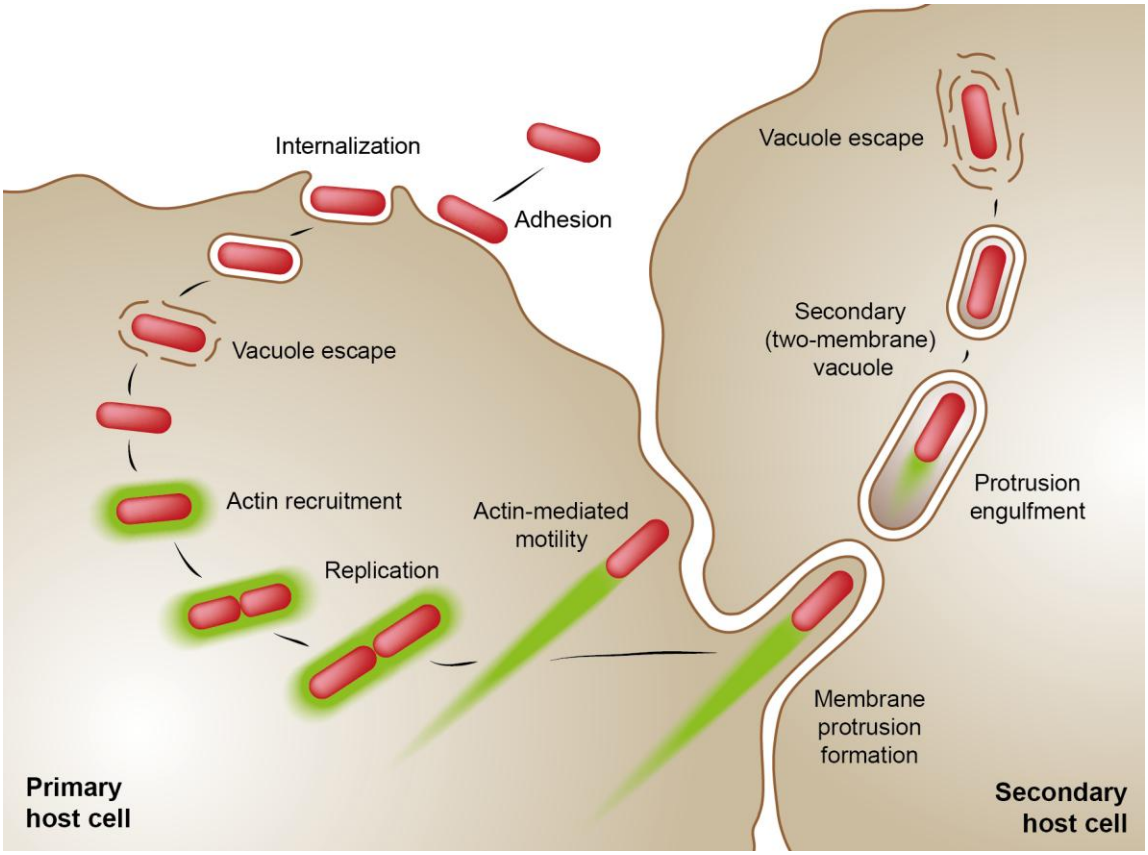

Fig. 3. Schematic representation of the successive steps of the *Lm* cellular infection cycle. *Lm* is depicted in red and host actin in green. (Adapted from Cossart and Toledo-Arana 2008)

engaging eukaryotic membrane receptors with invasion-promoting proteins (invasins) that trigger intracellular signaling cascades leading to a localized reshaping of the host cell cytoskeleton around the bacterium-cell interaction site. In a zipper-like fashion, *Lm* is gradually surrounded by host cell membrane and engulfed into an internalization vacuole. Soon after, aided by a secreted pore-forming toxin, *Lm* disrupts its containing vacuole and reaches the host cytoplasm, where a high nutritional availability favors bacterial replication. Once in this compartment, *Lm* cells begin to recruit host actin filaments that initially surround the whole bacterial surface (actin cloud) but later reassembles at one pole into a long comet-like tail (actin tail). Actin polymerization/depolymerization dynamics in this structure generate a propulsive force that confers random intracellular motility and allows *Lm* to eventually reach the cell membrane, forcing it into a protrusion that can be taken up by a neighboring uninfected cell. The resulting *Lm*-containing double-membrane secondary vacuole is rapidly lysed, enabling the bacterium to restart the infection cycle in a new cell without re-exposure to the extracellular environment (Fig. 3) (Vazquez-Boland *et al.* 2001, Cossart and Toledo-Arana 2008).

A.5.1. Major virulence factors

To successfully undertake each step of the host cell infection cycle, *Lm* is equipped with a highly diverse and evolutionarily perfected supply of virulence proteins, all of which are placed under the tight control of a complex, fine-tuned regulatory network (Camejo *et al.* 2011). In this section are described the most representative virulence factors involved in the different stages of the intracellular infection cycle.

Internalins A and B

Two members of the internalin family, internalin A (InlA) and B (InlB), were first bacterial proteins identified as mediators of *Lm* entry into host cells are (Gaillard *et al.* 1991, Dramsi *et al.* 1995). Members of this family contain a leucine-rich repeat (LRR) domain with variable length (Fig. 4) that is generally involved in

interaction with other proteins (Cabanes *et al.* 2002). Extensive functional characterization has strengthened their role as major listerial invasins.

InlA (800 aa) contains a second repeat region (B-repeat domain) that is separated from the LRR domain by an inter-repeat (IR) spacer region (Gaillard *et al.* 1991). In its C-terminal end, a cell wall-sorting signal region, containing an LPXTG motif, guides the covalent attachment of InlA to the peptidoglycan meshwork (Dhar *et al.* 2000) (Fig. 4). Together, the LRR and IR regions were shown to be indispensable and sufficient to support the entry of *Lm* into human epithelial cells (Lecuit 2007), as they form the minimal structure necessary to bind to the eukaryotic receptor for InlA, E-cadherin (E-cad) (Mengaud *et al.* 1996), a transmembrane glycoprotein expressed in epithelial cells and implicated in cell-cell adhesion. The InlA/E-cad interaction mimics the homotypal interaction between E-cad molecules from adjacent epithelial cells, which forms the basis of the tensile strength of adherens junctions that bind cells together. In this sense, the engagement of E-cad by InlA initiates a complex signaling pathway that activates a localized actin cytoskeleton rearrangement and ultimately leads to a clathrin-mediated internalization of the *Lm*-InlA/E-cad complex (Pizarro-Cerdá *et al.* 2012). Remarkably, variation of a single amino acid in E-cad dramatically changes host permissiveness to InlA-mediated infection, with humans and guinea pigs (E-cad$_{Pro16}$) showing susceptibility to orally inoculated *Lm*, whereas murinae (E-cad$_{Glu16}$) are resistant (Lecuit *et al.* 1999).

InlB (630 aa) displays a cell wall-anchoring C-terminal domain different from that of InlA, composed of multiple repeats that contain a conserved GW dipeptide (GW repeats) (Braun *et al.* 1997) (Fig. 4). These mediate the labile association with the *Lm* cytoplasmic membrane via non-covalent interactions with lipoteichoic acids (LTAs) (Jonquières *et al.* 1999), which results in co-existing surface-attached and secreted forms of InlB. In agreement with what was observed for InlA, the host cell invasive properties conferred by InlB (Braun *et al.* 1998) are also localized to the LRR domain (Braun *et al.* 1999). Unlike InlA, InlB has more than one interacting partner at the surface of eukaryotic cells. The most important is c-Met, a receptor tyrosine kinase known to bind hepatocyte growth factor (HGF). The role of this receptor in *Lm* infection was validated by showing that cells that did not express c-Met were resistant to InlB-mediated *Lm* entry (Shen *et al.* 2000).

Although some signaling players differ from those involved in the InlA-induced pathway, the *Lm* internalization mechanism activated by InlB/c-Met interaction similarly results in a reorganization of the actin network that promotes clathrin-mediated bacterial endocytosis (Pizarro-Cerdá *et al.* 2012). InlB was also shown to bind gC1qR, the receptor for the globular part of the C1q complement component (Braun *et al.* 2000), and glycosaminoglycans (GAGs) (Jonquières *et al.* 2001), both through its GW repeat domain (Jonquières *et al.* 2001, Marino *et al.* 2002). GAGs are able to sequester InlB molecules from the *Lm* surface and aggregate them around the host cell adhesion site, potentiating c-Met activation (Jonquières *et al.* 2001).

The diversified nature of their receptors and the differential cell- and tissue-specific expression result in a distinct cell tropism for *Lm* internalins: while InlA mostly promotes invasion of epithelial cells, such as those in the intestine and placenta (Gaillard *et al.* 1991, Lecuit *et al.* 2004), InlB mediates *Lm* entry into a wider variety of cell types, including hepatocytes (Dramsi *et al.* 1995), fibroblasts (Dramsi *et al.* 1997) and endothelial cells (Greiffenberg *et al.* 1998, Parida *et al.* 1998).

Listeriolysin O

To escape from the internalization vacuole, *Lm* secretes monomers of the pore-forming toxin listeriolysin O (LLO), a member of the family of cholesterol-dependent cytolysins (CDC) (Tweten *et al.* 2001), which oligomerize in the vacuole membrane as ring-like pore complexes (Shatursky *et al.* 1999, Tweten *et al.* 2001). LLO was the first *Listeria* virulence protein to be identified and functionally characterized in the context of infection. Mutants in the LLO-encoding gene, *hly* (for hemolysin), were drastically attenuated in virulence (>5 logs) in the mouse model (Gaillard *et al.* 1986, Kathariou *et al.* 1987). In cultured cells, they were unable to replicate because they remained trapped inside the vacuole (Gaillard *et al.* 1987, Portnoy *et al.* 1988), confirming the role of LLO in vacuolar membrane lysis. This role is not only confined to primary vacuoles, but also to the double-membrane secondary vacuole formed after *Lm* spreads from cell to cell (Gedde *et al.* 2000).

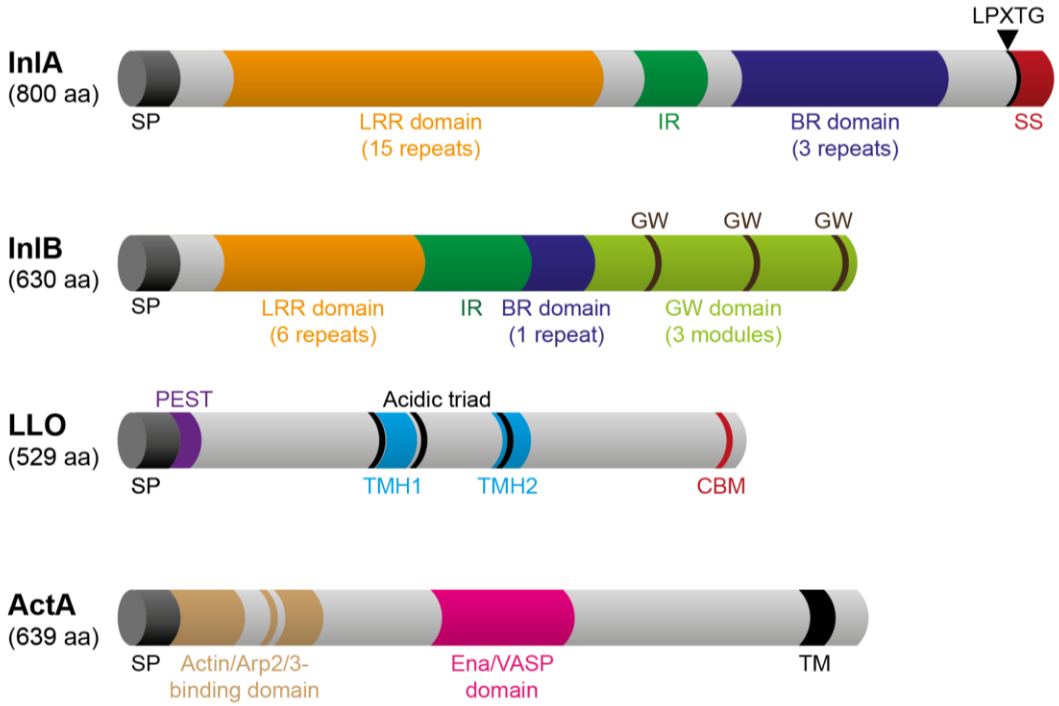

Fig. 4. Schematic representation of *Lm* virulence proteins InlA, InlB, LLO and ActA. Both InlA and InlB contain the signature internalin N-terminal LRR domain, which is followed by an IR region and a B-repeat (BR) domain. However, their C-terminal region is different: InlA has a sorting signal (SS) sequence with an LPTXG motif (enables covalent linkage to peptidoglycan), while InlB has GW dipeptide-containing module repeats (mediate non-covalent association with cell wall components). LLO contains an N-terminal PEST-like sequence, a central domain with two α-helices (TMH1 and TMH2) that span host cell membranes to form pores, and an acidic triad (Asp208, Glu247, Asp320) that mediates the pore-forming activity through pH-dependent conformational changes (Hamon *et al.* 2012); and a C-terminal cholesterol-binding motif (CBM). ActA is anchored to the membrane by a C-terminal transmembrane (TM) anchor and encodes its actin polymerization activity in two distinct domains: one recruits actin monomers and the actin nucleator Arp2/3 complex, while the other binds Ena/VASP family proteins that control actin filament assembly speed and direction. SP, signal peptide. (Adapted from Cabanes *et al*. 2002, Hamon *et al.* 2012, Köster *et al.* 2014 and Travier *et al.* 2013)

The cytolytic activity of LLO is optimal at a low pH (5.5) and lost almost completely at neutral pH (Geoffroy *et al.* 1987), explaining why the toxin is most active within the acidic vacuolar environment and loses its function upon *Lm* release into the cytoplasm (Beauregard *et al.* 1997). This pH-dependent regulation protects the host cell from further membrane damage, thus preserving an intracellular niche for *Lm* survival and proliferation (Glomski *et al.* 2003). An additional regulatory switch resides in the 5' coding region of the *hly* mRNA, encoding the N-terminal region of LLO. The presence of a PEST-like sequence within the LLO N-terminus (Fig. 4), suggested that it targeted LLO for cytosolic degradation (Rechsteiner and Rogers 1996, Decatur and Portnoy 2000). However, further studies denied this hypothesis (Lety *et al.* 2001) and implicated this *hly* mRNA region in translational repression of LLO during exponential growth of *Lm* (Schnupf *et al.* 2006), a situation verified in the host cell cytoplasm.

Other bacterial and host factors were shown to cooperate with the intravacuolar activity of LLO. Two bacterial proteins with phospholipase C (PLC) activity, PI-PLC and PC-PLC (encoded by the *Lm* virulence locus genes *plcA* and *plcB*), facilitate LLO-mediated escape from primary and secondary vacuoles, respectively (Smith *et al.* 1995), and in some cases, are able to mediate *Lm* escape in the absence of LLO (Marquis *et al.* 1995). Host proteins GILT and CFTR were also shown to support LLO function (Singh *et al.* 2008, Radtke *et al.* 2011).

A substantial body of evidence gathered in recent years has revealed additional roles for LLO in *Lm* infection other than vacuole rupture. Most of these novel functions are exerted extracellularly and are associated with signaling events: activation of NF-κB (Kayal *et al.* 1999), MAPK (Tang *et al.* 1996, Weiglein *et al.* 1997), calcium flux (Dramsi and Cossart 2003) and phosphoinositide metabolism pathways (Sibelius *et al.* 1996); downregulation of SUMOylation (Ribet *et al.* 2010); apoptosis of dendritic and T-cells (Guzman *et al.* 1996, Carrero *et al.* 2004); upregulation of cell adhesion molecules and cytokines (Yoshikawa *et al.* 1993, Nishibori *et al.* 1996, Kayal *et al.* 1999); mitochondrial fragmentation (Stavru *et al.* 2011) and histone modifications (Hamon and Cossart 2011).

ActA

Actin-mediated intracellular motility is a hallmark of the *Lm* cellular infection. The polymerization of actin filaments to form a polarized, dynamic tail structure with propulsive force is mediated by a 639-aa surface protein named ActA (Fig. 4) (Domann *et al.* 1992, Kocks *et al.* 1992). Encoded in the main *Lm* virulence locus (Vazquez-Boland *et al.* 1992), ActA alone was shown to be sufficient for recruitment of actin filaments (Pistor *et al.* 1994) and confer motility to otherwise non-motile bacteria (Kocks *et al.* 1995) and *Lm* mutants were non-motile in the host cell cytoplasm and avirulent in the mouse model (Domann *et al.* 1992, Kocks *et al.* 1992).

This protein is anchored to the bacterial membrane by a C-terminal transmembrane domain (Domann *et al.* 1992, Kocks *et al.* 1992), and contains two other domains responsible for actin filament-mediated motility. Near the N-terminus, three regions homologous to WASP protein sequences are essential for

actin filament polymerization and elongation (Lasa *et al.* 1997), through their recruitment of actin monomers and of the host actin nucleator Arp2/3 complex (Welch *et al.* 1998, Boujemaa-Paterski *et al.* 2001, Zalevsky *et al.* 2001). The presence of a proline-rich repeat (PRR) domain in the middle of ActA is not required for motility but is important for regulation of the actin filament tail speed and directionality (Fig. 4) (Lasa *et al.* 1995, Auerbuch *et al.* 2003). This domain binds members of the eukaryotic Ena/VASP protein family (Chakraborty *et al.* 1995), which not only recruit profilin, an actin monomer-binding protein (Theriot *et al.* 1994), but also modulate Arp2/3 complex activity by limiting filament branching and favoring the polymerization of parallel filaments (Samarin *et al.* 2003). A recent study demonstrated that the region between the Ena/VASP-binding domain and the transmembrane anchor is important for *Lm* aggregation and biofilm formation via ActA-ActA interactions, and that this activity if crucial for bacterial persistence in the intestinal tract (Travier *et al.* 2013).

Besides the pivotal role in *Lm* intracellular motility, ActA was also shown to be involved in other cellular infection events, such as epithelial cell invasion (Alvarez-Dominguez *et al.* 1997, Suarez *et al.* 2001), vacuole escape (Poussin and Goldfine 2010) and autophagy evasion (Yoshikawa *et al.* 2009).

B. GRAM-POSITIVE CELL ENVELOPE

The bacterial cell envelope is an elaborate, multilayered structure that provides structural support and protection from the external environment, while allowing exchange of nutrients and waste products. In Gram-negative organisms, this structure is composed of three concentric layers: a cytoplasmic (or inner) membrane, a peptidoglycan cell wall, and an outer membrane. In contrast, Gram-positive species lack an outer membrane but, in compensation, their peptidoglycan cell wall layer is significantly thicker to confer adequate resistance to turgor pressure and protection from external aggressions (Fig. 5) (Silhavy *et al.* 2010).

The work presented in this thesis is centered on *Listeria monocytogenes*, a Gram-positive pathogen. In accordance, this section describes the main components and features of this type of cell envelope.

B.1. Peptidoglycan

The presence of a cell wall layer made of peptidoglycan is a common characteristic to both Gram-negative and Gram-positive bacteria. However, unlike its Gram-negative homologue, the peptidoglycan cell wall is the major structural

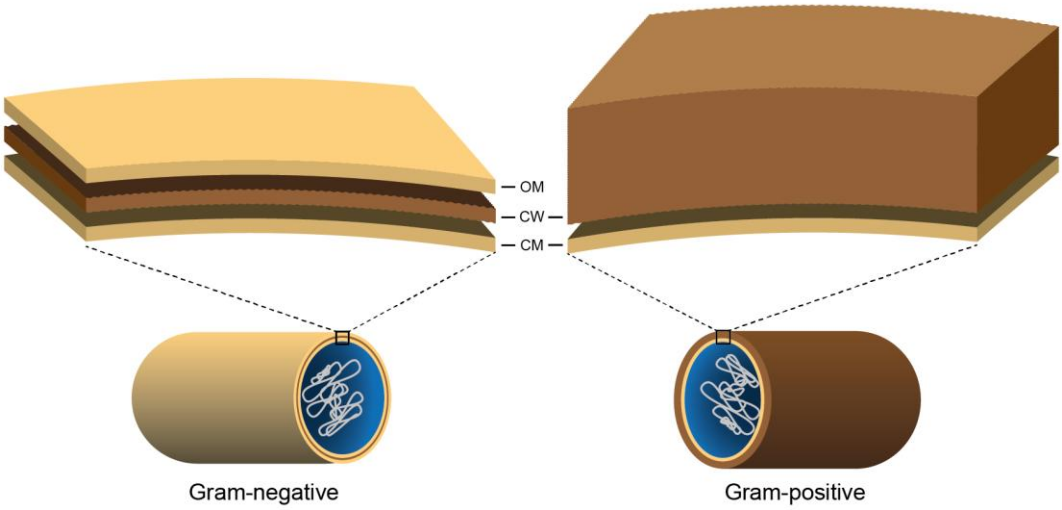

Fig. 5. Schematic representation of the basic cell envelope structure of Gram-negative and Gram-positive bacteria. Both bacterial classes possess a cytoplasmic membrane (CM) surrounded by a rigid cell wall (CW) layer. However, while the Gram-negative cell wall is conceiled by a second membrane (outer membrane, OM), the Gram-positive cell wall is the outermost surface layer and is significantly thicker.

component of the Gram-positive cell envelope, displaying a thickness of 30–100 nm with multiple connected layers (Silhavy *et al.* 2010). Additionally, it acts as a scaffold for the surface positioning of proteins and other glycopolymers with relevant physiological roles (Neuhaus and Baddiley 2003, Dramsi *et al.* 2008).

Peptidoglycan is a highly polymerized macromolecule composed of linear, parallel glycan strands linked perpendicularly by short peptide bridges (Fig. 6A). The glycan portion is constituted by alternating units of *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) linked by β(1–4) glycosydic bonds. The average glycan strand length is 50-250 Glc*N*Ac-Mur*N*Ac repeats (Ward 1973). The stem peptide element is linked to each Mur*N*Ac residue through its C3-linked lactoyl group and is typically constituted by the pentapeptide L-Ala-γ-D-Glu-N_2X-D-Ala-D-Ala. N_2X represents a diamino acid: L-Lys, in most Gram-positive species, or *meso*-2,6-diaminopimelic acid (*m*Dpm), in most Gram-negative species and Bacilli (including *Listeria*). The muropeptide Glc*N*Ac-Mur*N*Ac-pentapeptide constitutes the basic peptidoglycan subunit precursor (Fig. 6B) (Vollmer 2008).

The diamino acid residue is important for the cross-linkage between glycan strands, which occurs between its free (ε) amino group and the carboxyl group of the first D-Ala (position 4) of another stem peptide. In the case of *Lm* and other species with *m*Dpm-type peptidoglycan, the interpeptide linkage is a direct bond between *m*Dpm and D-Ala (Fiedler 1988), while a pentaglycine (Gly_5) bridge performs this role in L-Lys-type peptidoglycan (Fig. 6C). However, several other amino acid residues, stem peptide positions and interpeptide bridges have been catalogued by Schleifer and Kandler, who created a classification system for all these peptidoglycan types (Schleifer and Kandler 1972). According to this system, the *Lm* peptidoglycan belongs to the A1γ type (Kamisango *et al.* 1982).

As a result of the transpeptidation reaction, the terminal D-Ala is cleaved out in the mature peptidoglycan (Vollmer 2008). Additionally, the diamino acid is the acceptor anchor for covalently bound surface proteins (Dramsi *et al.* 2008).

B.1.1. Peptidoglycan metabolism

The continuous remodeling of the cell wall is paramount for bacterial growth and division, and requires a dynamic balance between peptidoglycan assembly

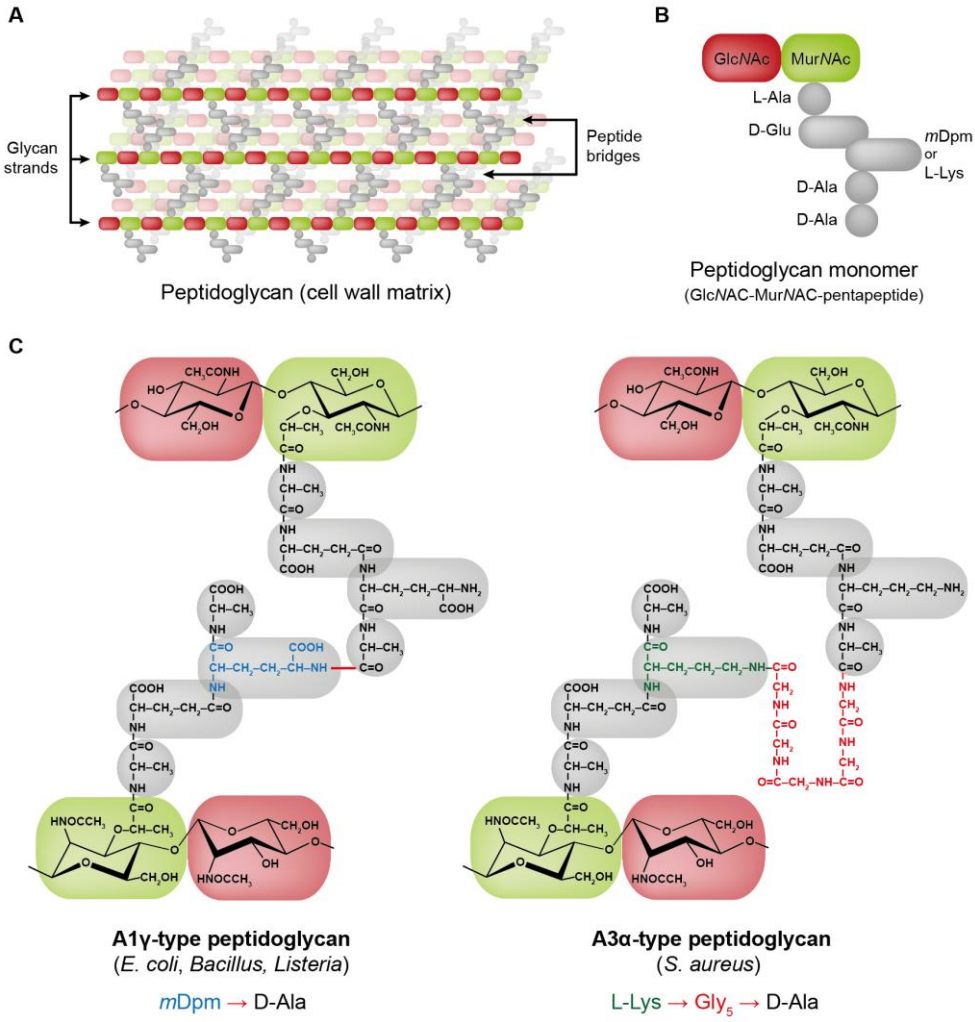

Fig. 6. Schematic representation of the peptidoglycan structure and the most common types of peptidoglycan strand cross-connections. (A) Peptidoglycan is a three-dimensional mesh-like structure composed of linear glycan strands connected between each other by peptide bridges. **(B)** Composition of a basic peptidoglycan monomer: a Glc*N*Ac-Mur*N*Ac disaccharide linked by the latter to pentapeptide stem containing typically L-Ala, D-Glu, a diamino acid (*m*Dpm or L-Lys), and two terminal D-Ala residues. In mature peptidoglycan, the last D-Ala is cleaved off during transpeptidation or by carboxypeptidases. **(C)** Common types of linkages between stem peptides from different glycan strands. In A1γ-type peptidoglycans, the ε-amino group of *m*Dpm (in blue) is directly linked to the carboxyl group of D-Ala in position 4. In *S. aureus* (A3α type), the ε-amino group of L-Lys (in green) is linked indirectly to D-Ala by a penta-glycine bridge (in red).

and turnover. Coordination between these processes is thus mandatory to prevent morphological malformations and concomitant functional defects, such as the mislocalization of surface molecules (Popowska 2004, Vollmer *et al.* 2008a).

B.1.1.1. Peptidoglycan assembly

Peptidoglycan is assembled outside of the bacterial cell through the polymerization of muropeptide subunits generated on the cytoplasmic side of the

membrane (van Heijenoort 1998). Following translocation, these building blocks are transferred and integrated into existing peptidoglycan chains by the action of a multifunctional family of surface proteins called penicillin-binding proteins (PBPs) (Fig. 7).

PBPs are membrane-anchored proteins that can be divided into high molecular weight (HMW) PBPs – the major players in peptidoglycan assembly – and low molecular weight (LMW) PBPs, both of which are characterized by the presence of an archetypal DD-peptidase domain (Macheboeuf *et al.* 2006). In HMW PBPs, the peptidase domain is located at the C-terminus and catalyzes transpeptidation reactions between adjacent glycan strands. Additionally, they may contain an N-terminal domain with transglycosylase activity, necessary for elongation of glycan strands (bifunctional PBPs). LWM PBPs perform roles linked to peptidoglycan maturation and recycling (Macheboeuf *et al.* 2006, Sauvage *et al.* 2008). The PBP peptidase domain recognizes the D-Ala-D-Ala moiety of immature stem peptides and cleaves the DD-bond. Penicillin and other β-lactam antibiotics take advantage of their structural similarity with the D-Ala-D-Ala dipeptide to bind irreversibly to and inhibit most PBPs, thus promoting bacterial death by perturbing cell wall synthesis (Tipper and Strominger 1965, Ghuysen 1994).

In silico studies have allowed the identification of ten PBP-like protein-encoding genes in the *Lm* genome (Guinane et al. 2006, Korsak et al. 2010), and β-lactam-binding assays confirmed that nine expressed functional PBPs (Korsak *et al.* 2010). They comprise five HMW proteins – class A members PBPA1 and PBPA2 (former PBP1 and PBP4), and class B members PBPB1, PBPB2 (former PBP3 and PBP2) and PBPB3 – and four LMW PBPs, including carboxypeptidase PBPD1 (former PBP5) and two β-lactamases (Korsak *et al.* 2010). Studies on listerial PBPs have largely focused on the determination of their affinity to several β-lactam derivatives (Gutkind *et al.* 1990, Pierre *et al.* 1990, Vicente *et al.* 1990, Guinane *et al.* 2006, Zawadzka-Skomial *et al.* 2006). In some cases, mutational approaches allowed the elucidation of the role of some PBPs towards *Lm* pathogenesis. For instance, PBPB1, PBPD1, but mostly PBPA2 and PBPC1, were found to be important for the colonization of the mouse spleen (Guinane *et al.* 2006). Depletion of these PBPs resulted in variable degrees of morphological defects (Guinane *et al.* 2006, Korsak *et al.* 2010), and the pleiotropic effects

elicited by such modifications are likely to be responsible for the attenuated virulence.

B.1.1.2. Peptidoglycan turnover

Peptidoglycan renovation relies on the activity of autolysins, another family of surface-associated enzymes that catalyze the hydrolysis of every existing covalent bond in the mature peptidoglycan matrix. The nature and location of the bond(s) cleaved by an autolysin is determined by its functional specificity within the broader family of peptidoglycan hydrolases (Vollmer *et al.* 2008b). *N*-acetylglucosaminidases (NAGases) and *N*-acetylmuramidases (NAMases) cleave the glycan strand β(1,4) bond after Glc*N*Ac and Mur*N*Ac, respectively. *N*-acetylmuramyl-L-alanine amidases (or simply amidases) separate the stem peptide from the glycan chain by breaking the bond between Mur*N*Ac and L-Ala. Finally, endopeptidases and carboxypeptidases hydrolyze the amide bonds within and between stem peptides (Vollmer *et al.* 2008b) (Fig. 7). The existence of multiple autolysins sharing the same activity and substrate attests for the functional redundancy associated with peptidoglycan hydrolases, a situation that has complicated the characterization of their individual role.

The genome of *Lm* strain EGD-e is predicted to encode six NAGases, four NAMases, four amidases, and a multiplicity of peptidoglycan peptidases, but only a few have been experimentally validated (Popowska 2004, Bierne and Cossart 2007, Pinto *et al.* 2013). The only predicted NAGases with confirmed peptidoglycan hydrolase activity are MurA and Auto, although their substrate specificity remains to be verified (Carroll *et al.* 2003, Cabanes *et al.* 2004). MurA is necessary for proper cell separation during growth and its absence or dysfunction results in virulence defects, namely in adhesion to host cells (Lenz *et al.* 2003, Alonzo *et al.* 2011). Auto is important for entry into non-phagocytic cells and virulence in mice and guinea pigs (Cabanes *et al.* 2004). The contribution of both autolysins towards *Lm* virulence possibly takes place through different mechanisms. This is suggested by their distinct cell wall association domains (MurA contains LysM repeats, Auto has GW modules; discussed below), which hint at a differential cell wall localization, and by their relative importance for cell

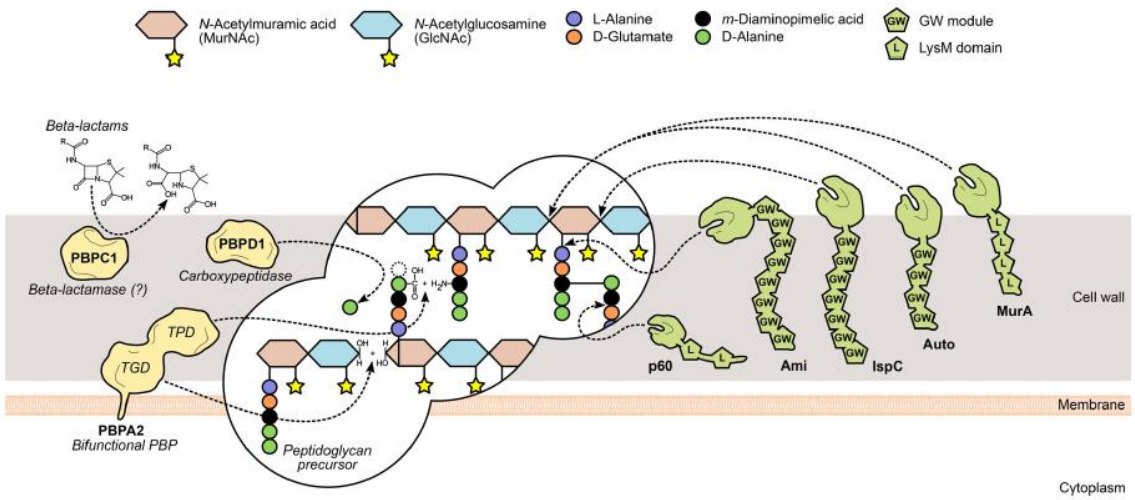

Fig. 7. *Lm* peptidoglycan metabolism and the surface proteins involved in its assembly and turnover. The peptidoglycan sacculus is polymerized from cytoplasmic precursors with the help of penicillin-binding proteins (PBPs, yellow). High-molecular-weight PBPs, such as PBPA2, contain transglycosylase (TGD) and transpeptidase domains (TPD) that catalyze, respectively, glycan chain elongation and stem peptide bridging between adjacent chains. Other PBPs include the low-molecular-mass carboxypeptidases, which cleave the terminal D-alanyl-D-alanine stem peptide bond (e.g. PBPD1), and beta-lactamases, which degrade PBP-inhibiting antibiotics to promote bacterial survival (e.g. PBPC1). On the other hand, the degradation of mature peptidoglycan, during bacterial elongation/division or autolysis, is mediated by autolysins (green), a family of surface hydrolases that can cleave the peptidoglycan at different sites: within the glycan chain (*N*-acetylglucosaminidases or *N*-acetylmuramidases) or the stem peptide (endo- and carboxypeptidases), or between both (*N*-acetylmuramoyl-L-alanine amidases). Interestingly, autolysins commonly associate non-covalently with the bacterial surface *via* cell wall-binding repeats, such as the GW modules in Ami, Auto and IspC, or the LysM repeats in MurA and p60. (Reproduced from Carvalho *et al.* 2014)

wall remodeling, since *murA* mutant cells cannot separate properly and grow in filaments, while *aut* mutants maintain a normal morphology (Carroll *et al.* 2003, Cabanes *et al.* 2004). Two putative *Lm* amidases contain C-terminal GW module repeats, suggesting similar surface association requirements; among them is the autolysin and virulence-promoting adhesin Ami (Milohanic *et al.* 2001).

Although none of the NAMases have been characterized in a virulence-oriented perspective, two were recently shown to possess lysozyme-like activity in the presence of cell wall substrate and to be required for stimulating the replication of quiescent bacteria, possibly through their impact in cell wall reshaping and thus in cell growth and division (Pinto *et al.* 2013). Nonetheless, IspC, a NAMase-like protein with a significant contribution to *Lm* infection, was identified in a serotype 4b strain (Wang and Lin 2007, 2008). Interestingly, IspC mutants were not affected in their growth *in vitro* and cell morphology, but showed cell type-dependent defects in nearly every step of the cellular infection cycle (Wang and Lin 2008).

The presence of an NlpC/p60 domain, related to the CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) superfamily is common to many

peptidoglycan hydrolases. Interestingly, most NlpC/p60 proteins are found in *Bacillus* and *Listeria*, but not in *Staphylococcaceae*, which express proteins with another CHAP-type domain (Bateman and Rawlings 2003, Layec *et al.* 2008). This is most likely a reflection of the affinity of the NlpC/p60 domain for the γ-D-Glu-*m*Dpm bond (Rigden *et al.* 2003), which is replaced by a γ-D-Glu-L-Lys linkage in staphylococci. Four *Lm* EGD-e proteins contain putative NlpC/p60 domains and were predicted to possess cell wall hydrolase activity (Bierne and Cossart 2007). Two of them, p45 (or Spl) and p60 (also CwhA or Iap), have been studied and their function validated. Spontaneous mutants secreting lower amounts of p60 showed a filamentous morphology and reduced host cell invasion efficiency, suggesting that p60 is required for entry into non-phagocytic cells. Indeed, exogenously added p60 not only restored *Lm* invasiveness (Kuhn and Goebel 1989), but also disrupted bacterial chains into individual cells, due to its cell wall-degrading activity (Wuenscher *et al.* 1993). Lack of functional p60 results in septum abnormalities that disrupt actin-based intracellular motility, impairing optimal cell-to-cell spread and, overall, virulence (Hess *et al.* 1996, Pilgrim *et al.* 2003, Faith *et al.* 2007).

B.2. Surface proteins and anchoring mechanisms

Proteins located at the bacterial cell surface carry out important and often vital functions, which – as described before – can be related with the interaction of the bacterium with its surrounding environment or with physiological events associated with cell surface maintenance or remodeling (e.g. growth/division). The correct localization of these proteins at the cell surface is therefore a requisite for proper activity.

In *Lm* and other Gram-positive bacteria, the cell wall is a preponderant component of the cell envelope and provides the main structural framework for protein anchoring (Navarre and Schneewind 1999). Protein-cell wall association can be established in two ways (Fig. 8): (i) stable covalent bonding between the peptidoglycan matrix and particular protein sorting motif sequences (LPXTG and NXXTX proteins), or (ii) labile, non-covalent interaction between cell wall components and cell wall-recognizing protein domains (LysM and GW proteins).

The cytoplasmic membrane also serves as a docking site for surface proteins, either directly through membrane-spanning domains (membrane proteins) or indirectly via a lipid anchor molecule (i.e. lipoproteins) (Fig. 8) (Cabanes *et al.* 2002, Desvaux *et al.* 2006).

B.2.1. Cell wall-associated proteins

B.2.1.1. LPXTG and NXXTX proteins

The precursors of proteins covalently anchored to the Gram-positive cell wall feature a C-terminal sorting signal sequence of about 30–40 residues comprising (i) an LPXTG pentapeptide motif (where X is any amino acid), followed by (ii) a hydrophobic domain, and (iii) a short positively charged tail (Schneewind *et al.* 1992). Whereas the hydrophobic and charged domains of the sorting signal can display variability in their sequence and/or length, the LPXTG motif is much conserved (Fischetti *et al.* 1990, Schneewind *et al.* 1992). Studies with C-terminal truncates of the staphylococcal protein A revealed that proper cell wall anchoring requires a complete sorting signal, and hinted that the hydrophobic and charged residues downstream of the LPXTG motif are responsible for retaining the polypeptide in the bacterial membrane until its recognition by a surface transpeptidase enzyme called sortase (Schneewind *et al.* 1992, Schneewind *et al.* 1993). The LPXTG motif is accommodated within the sortase active site, where a catalytic cysteine initiates cleavage of the peptide bond between the threonine and the glycine residues. The cleaved protein becomes temporarily bound to the sortase (Ton-That *et al.* 1999), which prevents its diffusion to the extracellular medium. The protein is then transferred to its final acceptor, lipid II (a membrane lipid-bound peptidoglycan precursor), where a new bond is formed between the free amine group of the stem peptide diamino acid residue (*m*Dpm in *Lm*) and the C-terminal threonine carboxyl group (Fig. 8) (Ton-That *et al.* 1997).

Proteins with LPXTG motifs are found in a multiplicity of Gram-positive organisms (Navarre and Schneewind 1999, Mazmanian *et al.* 2001, Hendrickx *et al.* 2009, Pérez-Dorado *et al.* 2012). However, *Lm* stands out as the species with the largest number, encoding 41 proteins (over 1% of its genome) (Glaser *et al.*

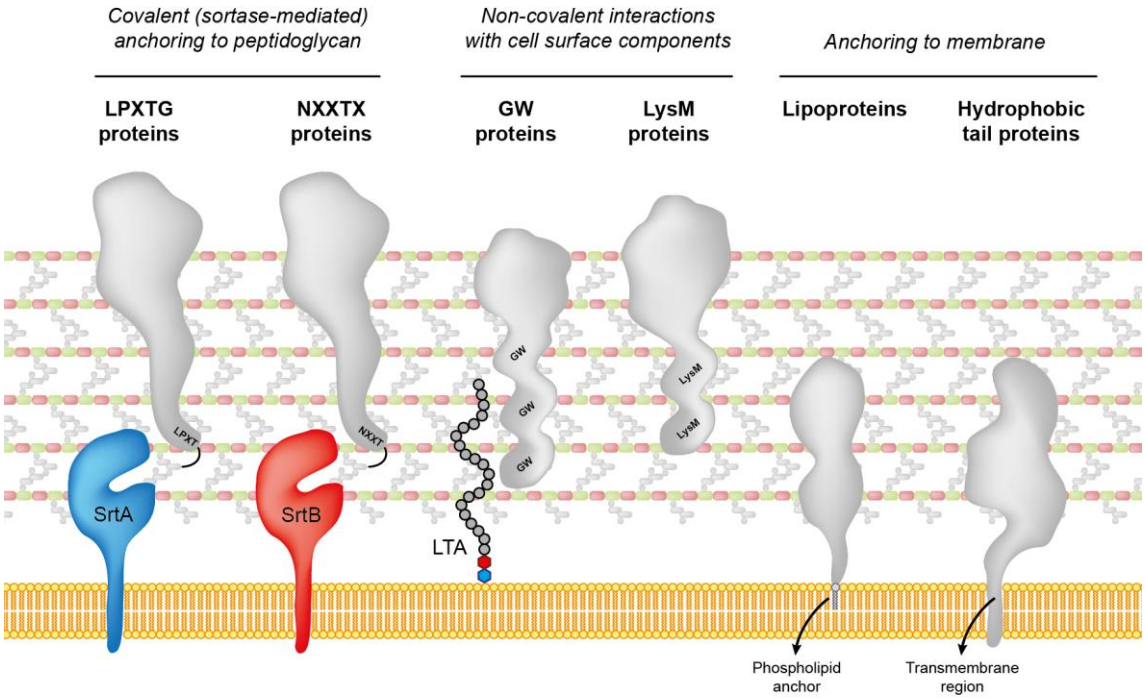

Fig. 8. Schematic representation of the main classes of surface proteins found in *Lm*. Proteins covalently associated to the peptidoglycan are processed by membrane transpeptidase enzymes called sortases, which recognize and cleave specific C-terminal sorting signal sequences (LPXTG or NXXTX) to append the mature protein to *m*Dpm residues in the peptidoglycan. All other proteins associate with the bacterial cell surface through non-covalent interactions that take place between cell wall-binding repeat domains (e.g. GW and LysM repeats) and cell envelope components (e.g. LTAs), or through protein tethering to the cytoplasmic membrane by means of N-terminally linked phospholipid anchors (lipoproteins) or short N- or C-terminal transmembrane regions rich in hydrophobic residues.

2001, Cabanes *et al.* 2002), seven of which are currently described as virulence factors (Table 2). InlA, important for entry into epithelial cells and virulence in mice (Gaillard *et al.* 1991, Lingnau *et al.* 1995), was the first to be identified, long before the *Lm* genome was sequenced. The list comprises four other internalin family members (Bierne *et al.* 2007) – InlF (Kirchner and Higgins 2008), InlH (Pucciarelli *et al.* 2005, Personnic *et al.* 2010), InlJ (Sabet *et al.* 2005, Sabet *et al.* 2008), and InlK (Dortet *et al.* 2011) – with roles in host cell adhesion and immune evasion, and two non-internalins – Vip (Cabanes *et al.* 2005) and LapB (Reis *et al.* 2010) – important for entry into cells.

A subset of covalently attached cell wall proteins feature a sorting signal different from that found in LPXTG proteins. This alternative signal is characterized by an NXXTX consensus sequence that targets surface protein precursors for processing by a second sortase, called sortase B to distinguish from the LPXTG-specific sortase or sortase A (Fig. 8) (Comfort and Clubb 2004, Mariscotti *et al.*

2009). Sortase B enzymes have fewer substrates, which are usually encoded by genes arranged in an operon together with the sortase B gene, *srtB* (Marraffini *et al.* 2006). Interestingly, they are involved in heme-iron scavenging and uptake (Mazmanian *et al.* 2002, Maresso and Schneewind 2006, Xiao *et al.* 2011, Klebba *et al.* 2012), indicating that the sortase B-mediated anchoring mechanism may have evolved differently from sortase A to become more specialized in the anchoring of proteins required for iron homeostasis.

Lm encodes only two proteins with NXXTX motifs (Table 2) (Bierne *et al.* 2004), both of which require sortase B for cell wall anchoring (Pucciarelli *et al.* 2005). One of them, SvpA (surface virulence protein A), is a surface-associated protein required for iron acquisition and persistence in mouse organs (Newton *et al.* 2005). The other, Lmo2186, possesses two putative sorting motifs, NKVTN and NPKSS (underlined residue is common to both), but only the latter is necessary for surface anchoring (Mariscotti *et al.* 2009). SvpA was first characterized as a virulence factor, as its absence resulted in deficient escape from macrophage phagosomes (Borezée *et al.* 2001). However, more recent data indicated that neither SvpA nor Lmo2186 are essential to promote infection (Newton *et al.* 2005), agreeing with results demonstrating that sortase B is dispensable for virulence (Bierne *et al.* 2004). Instead, they are implicated in heme scavenging under conditions of low iron availability, and are currently designated heme-binding proteins (Hbp) 2 and 1, respectively (Xiao *et al.* 2011).

Table 2. Examples of LPXTG and NXXTX proteins in *Lm*.

Protein	Gene	Size (aa)	Function	References
LPXTG proteins				
InlA	*lmo0433*	800	Host cell invasion	Gaillard *et al.* 1991; Lingnau *et al.* 1995
InlF	*lmo0409*	821	Host cell adhesion and invasion	Kirchner and Higgins 2008
InlH	*lmo0263*	548	Modulation of host inflammatory response (IL-6 production)	Personnic *et al.* 2010
InlJ	*lmo2821*	851	Host cell adhesion (*in vivo*)	Sabet *et al.* 2008
InlK	*lmo1290*	598	Autophagy evasion	Dortet *et al.* 2011
LapB	*lmo1666*	1711	Host cell adhesion and invasion	Reis *et al.* 2010
Vip	*lmo0320*	399	Host cell invasion	Cabanes *et al.* 2005
NXXTX proteins				
SvpA/Hbp2	*lmo2185*	569	Heme acquisition	Xiao *et al.* 2011
Hbp1	*lmo2186*	207	Heme acquisition	Xiao *et al.* 2011

B.2.1.2. LysM proteins

Lysin motif (LysM) domains are encountered in proteins from a broad variety of organisms, such as plants, fungi, bacteria, and viruses (Buist *et al.* 2008). Initially found in bacterial and phage lysins, from which the motif took its name (Birkeland 1994), the LysM domain is characterized by a variable number of roughly 40–80-residue repeats, spaced by stretches rich in serine, threonine, and asparagine (Buist *et al.* 1995). Their presence in proteins with cell wall-degrading activity suggested that LysM repeats are important for retention of these enzymes in the peptidoglycan (Fig. 8) (Joris *et al.* 1992, Birkeland 1994). This hypothesis was validated through binding studies using the LysM domains of *Lactococcus lactis* and *Enterococcus faecalis* autolysins (Steen *et al.* 2003, Eckert *et al.* 2006). Further studies singled out Glc*N*Ac as the peptidoglycan moiety bound by LysM (Buist *et al.* 2008). However, instead of an expected uniform surface distribution, many LysM-containing proteins appear localized to specific sites by the excluding action of cell wall components, such as lipoteichoic acids (Steen *et al.* 2003), or peptidoglycan modifications, such as *O*-acetylation (Veiga *et al.* 2007).

LysM domains are found in six *Lm* proteins (Bierne and Cossart 2007), among which are the p60 and MurA autolysins (Table 3) (Lenz *et al.* 2003). The N-terminal region of p60 contains two LysMs separated by a Src homology 3 (SH3)-like domain (Bierne and Cossart 2007), which presumably mediate binding to specific peptidoglycan sites important for p60 activity. In contrast, MurA contains four LysM repeats near its C-terminus (Carroll *et al.* 2003), which may be important to position the MurA catalytic site in a manner distinct of p60 to optimize its activity. A third LysM protein (Lmo2522) was recently characterized in *Lm* as one of two novel listerial resuscitation-promoting factors, i.e. muralytic enzymes important for jump-starting the growth of dormant bacteria (Pinto *et al.* 2013).

B.2.1.3. GW proteins

Many surface proteins interact non-covalently with the cell wall through a domain containing a variable number of tandemly arranged repeat sequences, called GW modules (Fig. 8). First discovered in the *Lm* invasion protein InlB

Table 3. LysM and GW proteins in *Lm*.

Protein	Gene	Size (aa)	Function	References
LysM proteins				
MurA/NamA	*lmo2691*	590	Autolysin (NAGase)	Carroll *et al.* 2003
p60/Iap	*lmo0582*	482	Autolysin (endopeptidase)	Kuhn and Goebel 1989; Wuenscher *et al.* 1993
Lmo2522	*lmo2522*	277	Autolysin (putative NAMase)	Pinto *et al.* 2013
Lmo0880	*lmo0880*	462	Unknown	
Lmo1303	*lmo1303*	109	Unknown	
Lmo1941	*lmo1941*	239	Unknown	
GW proteins				
InlB	*lmo0434*	630	Host cell invasion	Braun *et al.* 1997
Ami	*lmo2558*	917	Autolysin (Mur*N*Ac-L-Ala amidase)	Milohanic *et al.* 2000; Milohanic *et al.* 2001
Auto	*lmo1076*	572	Autolysin (NAGase)	Cabanes *et al.* 2004
Lmo1215	*lmo1215*	289	Autolysin (NAGase)[a]	Bierne and Cossart 2007
Lmo1216	*lmo1216*	328	Autolysin (NAGase)[a]	Bierne and Cossart 2007
Lmo1521	*lmo1521*	427	Autolysin (Mur*N*Ac-L-Ala amidase)[a]	Bierne and Cossart 2007
Lmo2203	*lmo2203*	375	Autolysin (NAGase)[a]	Bierne and Cossart 2007
Lmo2591	*lmo2591*	508	Autolysin (NAGase)[a]	Bierne and Cossart 2007
Lmo2713	*lmo2713*	312	Unknown	Bierne and Cossart 2007

a) Bioinformatic prediction from conserved domains

(Braun *et al.* 1997), its name derives from the presence of a conserved glycine (G)-tryptophan (W) dipeptide. InlB contains three GW modules in its C-terminal cell wall association domain that are required and sufficient to confer cell wall-binding properties to the protein (Braun *et al.* 1997). InlB variants lacking this domain are unable to associate to the surface of non-invasive *Listeria* and promote their entry into eukaryotic cells (Braun *et al.* 1998). Structural analysis of the GW module revealed an interesting resemblance with SH3 domains, known to be involved in protein-protein interaction in signal transduction pathways (Kaneko *et al.* 2008). However, steric hindrance issues discarded a functional SH3-like activity for GW modules (Marino *et al.* 2002).

The binding strength of proteins containing GW modules is proportional to the number of modules. This is illustrated by comparing the surface association levels of InlB and Ami, another GW protein with autolytic activity and an important role in bacterial adhesion to host cells (Milohanic *et al.* 2000, Milohanic *et al.* 2001, Asano *et al.* 2012). Containing eight GW modules, Ami is found exclusively in association with the bacterial surface, whereas InlB (only three modules) is detected in both cell envelope and in secreted protein fractions (Braun *et al.* 1997). *Lm* encodes seven other GW proteins (Table 3), all of which have a

predicted amidase domain in common with Ami (Cabanes *et al.* 2002), hinting that they also may possess autolytic functions. Indeed, one of them, Auto, was described to also function as an autolysin (Cabanes *et al.* 2004). Staphylococcal autolysins are also associated to the bacterial surface by structural motifs resembling listerial GW modules (Oshida *et al.* 1995, Heilmann *et al.* 1997, Hell *et al.* 1998, Allignet *et al.* 2001), strongly suggesting that this cell wall association protein motif has evolved with the purpose of mediating the reversible surface binding of proteins with autolytic activity (Milohanic *et al.* 2001).

Lipoteichoic acids (LTAs) were identified as the InlB surface anchor, binding to its cell wall association domain. The interaction with these cell envelope glycopolymers is highly specific, as LTAs from *L. innocua* or *S. pneumoniae* are not able to capture InlB (Jonquières *et al.* 1999). The cell wall association domain of InlB also mediates its interaction with GAGs present at the surface of host cells and with gC1qR, significantly potentiating InlB-mediated invasion (Braun *et al.* 2000, Jonquières *et al.* 2001, Banerjee *et al.* 2004, Asano *et al.* 2012).

B.2.2. Membrane-associated proteins

B.2.2.1. Lipoproteins

Bacterial lipoproteins contribute to important physiological roles, such as substrate binding and transport, antibiotic resistance, signaling, and protein folding (Sutcliffe and Russell 1995, Hutchings *et al.* 2009). They were also shown to take an active part in virulence-associated processes, such as adhesion, invasion, and immunomodulation (Kovacs-Simon *et al.* 2011, Nakayama *et al.* 2012).

Lipoproteins are expressed as immature polypeptides, which are converted to prolipoproteins by the addition of a lipid moiety at a specific motif in the distal portion of the N-terminal signal peptide. This motif, called lipobox, is characterized by a four-residue sequence containing a conserved cysteine (Sutcliffe and Harrington 2002, Babu *et al.* 2006). The sulfhydryl group of the cysteine establishes a thioester bond with phospholipid-derived diacylglycerol, in a reaction catalyzed by the Lgt transferase (Kovacs-Simon *et al.* 2011). The N-terminal lipid

anchor inserts into the outer leaflet of the bacterial cytoplasmic membrane (Fig. 8), enabling the surface retention of the protein upon signal peptide cleavage.

In *Lm*, the biological importance of lipoproteins is emphasized by their preponderance in the surface proteome: 68 of 133 surface proteins were predicted to be lipoproteins, based on the presence of an N-terminal lipobox (Glaser *et al.* 2001), and 26 were later confirmed experimentally (Baumgärtner *et al.* 2007). Interestingly, nearly half of listerial lipoproteins are presumed to act as substrate-binding components of ABC transporter systems (Bierne and Cossart 2007), performing the equivalent functions of periplasmic solute-binding proteins in Gram-negative bacteria (Tam and Saier 1993). This is the case of lipoproteins OppA, which participates in oligopeptide uptake, and LpeA, which belongs to the LraI family of manganese-importing ABC transporter components (Novak *et al.* 1998), although supporting evidence for this function in *L. monocytogenes* have yet to be obtained. Another substrate-carrying lipoprotein, OpuC, operates in the transport of L-carnitine, important for *Lm* osmotolerance and persistence in mice organs (Sleator *et al.* 2001). Fifteen other *Lm* lipoproteins were predicted to perform enzymatic roles (Bierne and Cossart 2007). Among them, the best studied and with a significant contribution to infection is the surface chaperone PrsA2 (Chatterjee *et al.* 2006, Alonzo *et al.* 2009, Zemansky *et al.* 2009, Forster *et al.* 2011).

B.2.2.2. Hydrophobic tail proteins

Surface proteins can be associated with the bacterial cytoplasmic membrane through an N- or C-terminal tail region comprised of hydrophobic amino acid residues that spans and stably inserts the protein in the lipid bilayer, during translocation (Fig. 8). The orientation of the proteins in the membrane is pre-determined by the presence and localization of positively charged residues relative to the membrane-spanning domain (stop-transfer signals) (Dalbey *et al.* 2011).

From the ten predicted *Lm* surface proteins with a putative C-terminal hydrophobic tail (Bierne and Cossart 2007), only ActA has been biochemical and functionally characterized (Domann *et al.* 1992, Kocks *et al.* 1992). A large number of listerial enzymes linked with cell wall metabolism and surface protein processing

– e.g. sortases (Mazmanian *et al.* 2000), signal peptidases (Paetzel *et al.* 2000) and PBPs – are anchored to the bacterial membrane by an N-terminal hydrophobic tail (Bierne and Cossart 2007), which in many cases corresponds to a signal peptide sequence lacking a cleavage site recognized by a signal peptidase.

B.2.3. Proteins with unknown association mechanism

Several proteins secreted by *Lm* lack any recognizable surface-targeting sequences. Moreover, a part of these proteins is associated with the cell envelope despite having no predicted surface-binding domains (Schaumburg *et al.* 2004, Trost *et al.* 2005). Consistent and, in some cases, significant secretion of the same proteins in different studies seems to discard or at least minimize the contribution of bacterial cell lysis to their extracytoplasmic localization. In turn, it suggests that they use a non-classical type of secretion mechanism (Schaumburg *et al.* 2004). So far, FbpA is the only known example of an unconventionally secreted and surface-associated protein with a described virulence-promoting function. Like many streptococcal fibronectin-binding proteins, FbpA lacks all the classical cell surface sorting and anchoring sequences. However, the protein was still detected in the *Lm* cytoplasmic membrane after subcellular fractionation. FbpA was found to facilitate adhesion to hepatocytes *in vitro* and to support liver infection in mice (Dramsi *et al.* 2004).

B.3. Teichoic acids

In addition to surface proteins, the Gram-positive peptidoglycan is densely decorated with different families of secondary glycopolymers, which include teichoic acids, teichuronic acids, and S-layer protein-associated glycans (Weidenmaier and Peschel 2008). From these, only teichoic acids are synthesized in *Listeria*, where they make up to 60% of the dry cell wall mass (Fiedler *et al.* 1984).

First discovered in 1958 by Baddiley and colleagues (Armstrong *et al.* 1958), teichoic acids (TAs; from the Greek *teichos*, i.e. “wall”) are described as a broad family of surface glycopolymers constituted by phosphodiester-linked polyol

subunits that can also contain glycosyl or D-alanyl ester groups (Baddiley 1970, Ward 1981). The abundance of phosphate groups confers strong anionic properties to TAs, which contribute to the net negative charge of the bacterial surface. This electrostatic status is fundamental for cell envelope-related processes, such as cationic homeostasis (required for optimal activity of surface proteins) and trafficking of nutrients, proteins and antibiotics (Neuhaus and Baddiley 2003). Moreover, the presence of TAs at the Gram-positive cell surface is also important for the targeting and anchoring of surface proteins (e.g. autolysins), with impact in cell growth and division; for providing protection against antimicrobial compounds, and for host-pathogen interactions (Weidenmaier and Peschel 2008).

Depending on their type of linkage to the cell surface, TAs are distributed into two groups: lipoteichoic acids (LTAs) and wall teichoic acids (WTAs).

B.3.1. Lipoteichoic acids (LTAs)

B.3.1.1. LTA structure and biogenesis

LTAs comprise a structurally diverse group of TAs (Neuhaus and Baddiley 2003) that are attached to the outer leaflet of the bacterial membrane through a glycolipid anchor, which consists of a sugar moiety (di- or oligosaccharide) linked to diacylglycerol (DAG) (Fischer *et al.* 1990). In representative Firmicutes species like *B. subtilis*, *S. aureus* and *Lm*, the LTA backbone comprises a linear chain of 1,3-linked glycerol 1-phosphate (GroP) repeats that are variably substituted with D-alanyl and/or glycosyl residues at the C2 hydroxyl group (Fig. 9A) (Fischer *et al.* 1990). Multiple analytical studies on the LTA composition have indicated an average chain length of 17–27 GroP units (Hether and Jackson 1983, Uchikawa *et al.* 1986b, Fischer 1994). Whereas these bacteria share the same LTA backbone composition, their glycolipid anchors show variation in the sugar moiety: glucose-glucose (Glc-Glc)-DAG (*B. subtilis* and *S. aureus*) or galactose-glucose (Gal-Glc)-DAG (*Lm*, Fig. 9A) (Uchikawa *et al.* 1986b, Jorasch *et al.* 2000, Kiriukhin *et al.* 2001). The anchor is synthesized in the cytoplasmic side of the bacterial membrane and translocated to the extracellular side to accept GroP subunits

derived enzymatically from outer leaflet membrane phospholipids for polymerization of the LTA backbone (Reichmann and Gründling 2011). In contrast to *S. aureus* and *B. subtilis*, which use respectively one and up to four enzymes to synthesize the LTA backbone (Reichmann and Gründling 2011), *Lm* requires two enzymes: one (LtaP) to prime the anchor with the first GroP molecule, and the other (LtaS) to extend the chain with further GroP subunits (Fig. 9B). Only LtaS is essential for LTA synthesis, and its depletion results in temperature-sensitive growth and defects in cell shape and septal division (Webb *et al.* 2009).

B.3.1.2. LTA modifications and functions

Modifications of the LTA backbone, such the addition of D-alanine or glycosyl groups, are carried out outside the cell by enzymatic complexes that translocate the cytoplasmic substrates across the membrane and append them to the LTA chain (Percy and Gründling 2014).

LTA D-alanylation is catalyzed by the products of the *dltABCD* operon (Perego *et al.* 1995, Neuhaus and Baddiley 2003, Reichmann *et al.* 2013), and the resulting D-alanyl ester linkage is highly sensitive to changes in temperature, pH and salt concentration (Hurst *et al.* 1975, Fischer and Rosel 1980, Macarthur and Archibald 1984). Mutational studies in different bacteria have highlighted the importance of this LTA modification in physiological functions, such as regulation of autolysis (Steen *et al.* 2005, Fedtke *et al.* 2007) and cation homeostasis (Archibald *et al.* 1973). Importantly, it also plays an essential role in bacterial pathogenesis, as demonstrated by the reduced host cell adhesion and virulence levels of *dltA* mutants of *Lm* (Abachin *et al.* 2002). Interestingly, the increased adhesion of *dltA* mutants of *Enterococcus faecalis* to uroepithelial cells suggests that the contribution of this LTA modification towards infection is cell type-dependent (Wobser *et al.* 2014). In addition, D-alanylation of LTAs was also shown to provide significant protection against cationic antimicrobial peptides (CAMPs) (Abachin *et al.* 2002). This protective mechanism is based on the reduction of the cell envelope net negative charge by the addition of positively charged D-alanyl groups, thus decreasing CAMP affinity for the bacterial surface (Peschel *et al.* 1999).

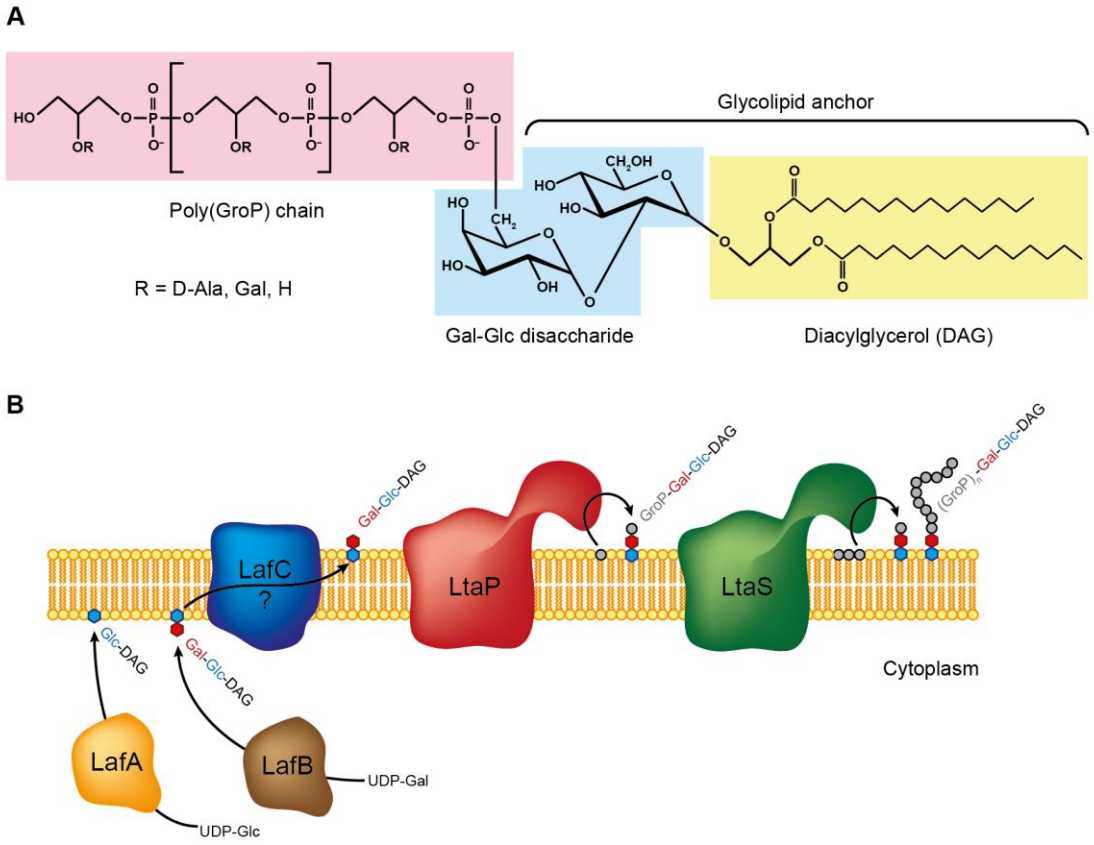

Fig. 9. Structure and biosynthesis pathway of *Lm* LTAs. (A) LTA polymers are composed of a poly(glycerol-phosphate) backbone chain (pink) that is attached to the cytoplasmic membrane via a glycolipid anchor consisting of a Gal-Glc disaccharide (blue) linked to a phospholipid-derived diacylglycerol molecule (yellow). The backbone subunits can be substituted with D-Ala or Gal. **(B)** LTA biosynthesis starts with the glycolipid anchor assembly on the inner leaflet of the cytoplasmic membrane by the concerted action of LafA and LafB. The anchor is then translocated to the extracellular side (presumably by the LafC transmembrane protein), where a phospholipid-derived GroP molecule is transferred to the disaccharide by LtaP. Additional GroP units are introduced by a second protein, LtaS, which is essential for LTA biogenesis. UDP, uridine diphosphate. (Adapted from Reichmann and Grundling 2011)

The mechanism and role of LTA glycosylation are still poorly understood topics. What is known so far is that the occurrence, degree and type of glycosyl residues used in this modification can vary between species and even between strains (Iwasaki *et al.* 1986, Iwasaki *et al.* 1989). Interestingly, *Listeria* strains were found so far to be only glycosylated with Gal (Hether and Jackson 1983, Uchikawa *et al.* 1986b). Although the enzymatic players have not yet been identified, it is hypothesized that cytoplasmic nucleotide-activated sugars are captured by a glycosyltransferase, which transfers the sugar moiety to a membrane lipid anchor; this sugar-lipid complex is translocated across the membrane and recognized by a second glycosyltransferase, which picks up the sugar and links it to the LTA backbone (Percy and Gründling 2014).

Numerous evidences of morphological and septal formation phenotypes associated with LTA mutants have implied the participation of these surface glycopolymers in cell growth/division-related processes. A recent study in *S. aureus* confirmed this by demonstrating a direct interaction between the LTA biosynthesis and cell division protein machineries (Reichmann *et al.* 2014). LTAs have also potent immunostimulatory properties (Morath *et al.* 2001). They are known to interact with and activate Toll-like receptor (TLR) 2, for which only the glycolipid anchor and a few backbone subunits are required (Deininger *et al.* 2003). This activation was shown to be stronger in the presence of D-alanylated subunits (Deininger *et al.* 2007). In addition, LTAs are specifically recognized by innate immunity lectin-like proteins such as L-ficolin, one of components of the lectin pathway of complement system activation (Lynch *et al.* 2004), and surfactant protein D, an innate immunity mediator in the lung (van de Wetering *et al.* 2001).

B.3.2. Wall teichoic acids (WTAs)

B.3.2.1. WTA structure and biogenesis

WTAs are covalently anchored to the cell wall, where they make up as much as 60% of its carbohydrate composition (Baddiley 1972, Neuhaus and Baddiley 2003). They are attached to the peptidoglycan by a linkage unit that contains a conserved GroP-*N*-acetylmannosamine (Man*N*Ac)-β(1,4)-Glc*N*Ac triad linked by a phosphodiester bond to the C6 hydroxyl group of Mur*N*Ac residues (Araki and Ito 1989). In *Lm*, the first WTA backbone subunit is connected to the conserved linkage unit via a Glc-Glc bridge, which is thought to further distance the main chain from the peptidoglycan (Fig. 10A) (Kaya *et al.* 1985).

WTAs are biochemical and structurally heterogeneous polymeric complexes, a diversity that is mainly observed in the type of backbone monomers and glycosyl substituent groups (Naumova *et al.* 2001). The most common WTA backbone (type I) comprises a linear chain of phosphodiester-linked repeats of either glycerol 3-phosphate (GroP) or ribitol 5-phosphate (RboP) (Brown *et al.* 2013), although other polyols can also be found (Naumova *et al.* 2001). Backbone length can surpass 40 units, which depending on the attachment point in the cell wall may

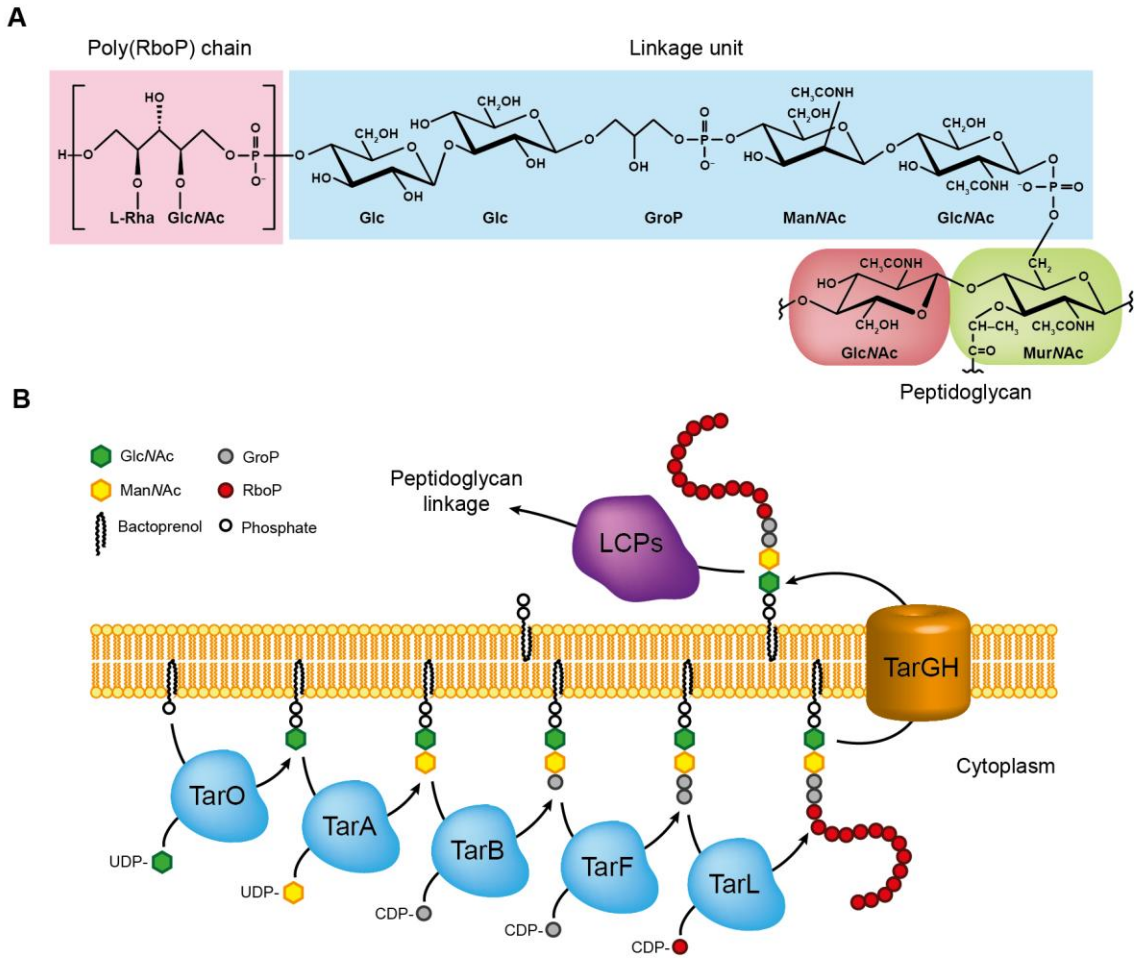

Fig. 10. Structure of *Lm* WTAs and representative biosynthesis pathway from *S. aureus*. (A) *Lm* WTA polymers are composed of a poly(ribitol-phosphate) backbone chain (pink) that is attached to the peptidoglycan matrix (Mur*N*Ac residues) via a unique linkage unit (blue). The backbone subunits are substituted with Glc*N*Ac and L-rhamnosyl (L-Rha) groups. **(B)** The WTA biosynthetic pathway in *Lm* has not been specifically addressed and characterized in detail, but it is assumed closely similar to that of *S. aureus*, which also produces RboP-type WTAs. The WTA polymer is sequentially assembled by the Tar enzymes onto a bactoprenol-phosphate anchor on the inner leaflet of the cytoplasmic membrane. Backbone substitution with glycosyl groups also takes place in the cytoplasm before the lipid-anchored polymer is translocated across the membrane by the TarGH complex. Outside, a set of WTA ligases (LCPs in *S. aureus*) cleave the polymer from its lipid anchor and mediate its attachment to the peptidoglycan matrix. (Adapted from Swoboda *et al.* 2010)

allow WTAs to extend beyond the cell envelope surface (Umeda *et al.* 1992). Studies have shown that while some species can only produce WTAs with a single type of polyol (e.g. RboP in *S. aureus* and *Lm*), others are able to use different types (e.g. *B. subtilis*: strain 168, GroP; strain W2, RboP) (Baddiley *et al.* 1961, Kamisango *et al.* 1983, Iwasaki *et al.* 1986).

Although structurally similar, WTAs have a biosynthetic pathway completely distinct from that of LTAs (Ward 1981) and which has been best characterized in

B. subtilis and *S. aureus*. The WTA polymer is synthesized at the inner leaflet of the cytoplasmic membrane, on top of undecaprenyl-phosphate (C55-P, also called bactoprenol-phosphate), a membrane-associated lipid anchor that is also recruited by the peptidoglycan biosynthesis machinery (van Heijenoort 1998). The linkage unit is the first component to be assembled through a pathway highly conserved across Gram-positive bacteria (Fig. 10B). It begins with the transfer of Glc*N*Ac-1-phosphate from cytoplasmic UDP-Glc*N*Ac to C55-P in a step catalyzed by the TagO/TarO enzyme (Soldo *et al.* 2002). Interestingly, two TagO orthologues with redundant activity were recently identified in the *Lm* genome (Eugster and Loessner 2012). Subsequent TagA/TarA-mediated binding of Man*N*Ac to Glc*N*Ac commits the lipid-anchored intermediate towards WTA bionsynthesis, and TagB/TarB concludes linkage unit synthesis by transfering a GroP molecule from CDP-glycerol to Man*N*Ac (Ginsberg *et al.* 2006). Afterwards, the WTA biosynthetic pathways diverge depending on whether the polymer is built with RboP or GroP monomers. In each case, the required enzymes are encoded by genes usually organized in operons and prefixed with the *tar* (teichoic acid ribitol) or *tag* (teichoic acid glycerol) designations, respectively (Brown *et al.* 2013). It is unclear if WTAs cross the membrane during or after their polymerization or how their translocation through the Ta(g/r)GH complex is processed, but once the polymer is transferred to the other side of the membrane, a family of WTA ligases (LCP proteins in *S. aureus*) promotes their attachment to the peptidoglycan (Fig. 10B) (Chan *et al.* 2013).

B.3.2.2. WTA modifications and functions

Similarly to LTAs, the backbone of WTAs can be tailored with D-alanyl esters and/or glycosyl groups. In the case of Rbo-type WTAs, which have three available hydroxyl groups, D-alanine binds to the C2 hydroxyl group, while *O*-glycosylation occurs at position C4 (Neuhaus and Baddiley 2003). Unlike D-alanine residues, which are bound to the polymer only after it is translocated to the extracellular space (Neuhaus and Baddiley 2003), sugar substitution is performed in the bacterial cytoplasm by a variable number and type of glycosyltransferases (Brown *et al.* 2013). As in LTAs, the WTA content in D-alanine is highly dependent of

environmental changes (Neuhaus and Baddiley 2003), in stark contrast with the stability of the sugar substituents (Collins *et al.* 2002). A recent study suggesting the requirement of LTAs for the D-alanylation of WTAs (Reichmann *et al.* 2013) appears to substantiate a previously proposed model whereby the D-alanyl groups of WTAs are donated by LTAs (Haas *et al.* 1984). However, the mechanism and putative enzymes involved in this transfer still need to be addressed.

A wide variety of glycosyl residues can be found associated to WTA subunits as branching substituent groups or even integrated in the main chain (non-type I WTAs): Glc, Gal, L-rhamnose (L-Rha), and *N*-acetylated amino sugars (Naumova *et al.* 2001). Further WTA heterogeneity can arise from the stereochemical nature of the glycosidic bond (Nathenson *et al.* 1966), which can interfere with WTA structure and its potential functions and interactions at the cell envelope.

The structural and biochemical similarities between WTAs and LTAs bring about a considerable functional overlap and redundancy that has complicated a clear understanding of the exact role of each glycopolymer in different aspects of bacterial physiology. As LTAs, WTAs are also intimately related with cell growth and division processes, as mutants lacking these polymers present morphological defects (Brown *et al.* 2013). Indeed, co-localization of WTA and peptidoglycan biogenetic machineries was observed (Formstone *et al.* 2008), and evidence showing mislocalization of important septal peptidoglycan cross-linking enzymes in the absence of WTAs (Atilano *et al.* 2010, Qamar and Golemi-Kotra 2012) attest this functional relationship. In addition, WTA depletion also interferes with cell division as it deregulates autolytic activity at the septum, indicating that WTAs are essential in regulation of autolysis, either by controlling autolysin localization or their activity (Brown *et al.* 2013).

Other WTA functions shared with LTAs include the regulation of cationic homeostasis in the cell envelope, where WTAs extending beyond the cell wall surface can act as cation “scavengers”, capturing these ions from the extracellular environment (Kern *et al.* 2010); protection against antimicrobial molecules (e.g. fatty acids, antibiotics and cationic peptides) (Peschel *et al.* 1999, Peschel *et al.* 2000, Collins *et al.* 2002, Kohler *et al.* 2009, Brown *et al.* 2012, Farha *et al.* 2013); and pathogenesis (Collins *et al.* 2002, Weidenmaier *et al.* 2004, Kristian *et al.* 2005, Weidenmaier *et al.* 2005, Walter *et al.* 2007). In most, if not all, backbone

modification with D-alanine performs a central role.

Interestingly, unlike *S. aureus*, *Lm* WTAs are not D-alanylated (Fiedler 1988), which suggests that functions associated with this modification are performed by LTAs and casts even more uncertainty on the actual WTA-specific functions. On the other hand, glycosylation was shown to confer immunogenic properties to *Lm* WTAs (Kamisango *et al.* 1983) and enable the binding of bacteriophages (Wendlinger *et al.* 1996), as previously observed (Juergens *et al.* 1963, Torii *et al.* 1964, Chatterjee *et al.* 1969). Evidences linking WTA glycosylation with *Lm* pathogenesis were obtained from studies with transposon-generated mutants. EGD (serotype 1/2a) mutants were screened in a mouse model for virulence attenuation (Autret *et al.* 2001), and multiple attenuated clones were found to contain an insertion in *gtcA*, a gene coding for a glycosyltransferase responsible for the tailoring of serotype 4b and 1/2a WTAs with Gal and Glc*N*Ac, respectively (Promadej *et al.* 1999, Eugster *et al.* 2011). In another study, the pathogenic potential of a serotype 4b *gtcA* mutant was strongly reduced in intragastrically infected mice. Moreover, the absence of GtcA decreased the ability of *Lm* to invade an enterocytic cell line, suggesting that GtcA-mediated WTA glycosylation is important for the intestinal phase of listeriosis (Faith *et al.* 2009).

B.3.2.3. WTA diversity in *Listeria monocytogenes*

In 1969, Ullmann and Cameron described the immunochemical properties of cell wall carbohydrates isolated from several *Lm* serotypes and their main antigenic constituents (Ullmann and Cameron 1969). As TAs began being regarded as *O* antigens, further research from groups in Germany and Japan, during the 1980s, led to the characterization of the WTA structure, composition and properties from every known *Lm* serotype (Kamisango *et al.* 1983, Fiedler *et al.* 1984, Fujii *et al.* 1985, Uchikawa *et al.* 1986a). Their extensive work revealed the large variety of glycosylation patterns across different serotypes and even within serotypes from the same serogroup (Fig. 11). It is this significant WTA tailoring diversity that constitutes in part the basis for serotyping classification within this species.

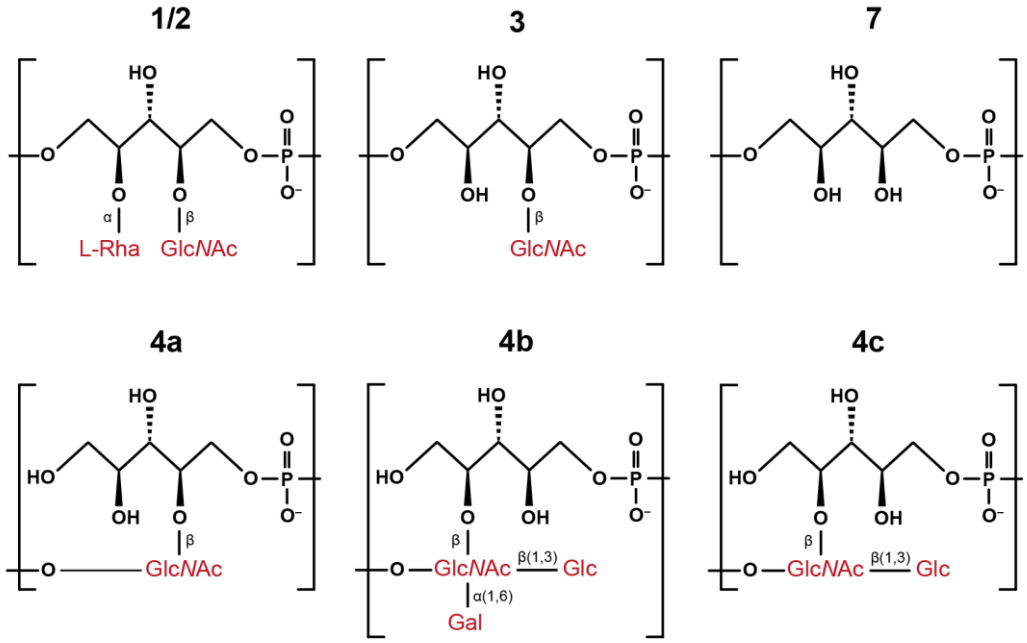

Fig. 11. WTA backbone subunit composition from different *Lm* serotypes. WTAs from serogroups 1/2 (1/2a, 1/2b and 1/2c) and 3 (3a, 3b and 3c), and serotype 7 have a typical poly(RboP) backbone, which can be variably substituted with Glc*N*Ac and/or L-Rha, or unsubstituted at all. The backbone subunits of serogroup 4 (4a, 4b and 4c) WTAs are more complex, as a Glc*N*Ac residue is integrated into the chain, forming a poly(RboP-Glc*N*Ac)-type polymer, and substitution with Glc and/or Gal occurs on this sugar instead of RboP.

C. ANTIMICROBIAL PEPTIDES

It was known since the late 19th century that many human secretions and fluids exhibited antimicrobial properties, which were later associated with particular peptides and small proteins present in their composition (Skarnes and Watson 1957). In the first half of the 20th century, several peptide antibiotics were identified and began being isolated from bacteria and fungi for clinical purposes (Perlman and Bodanszk.M 1971). In the 1950s, these antimicrobial peptides (AMPs) were thought to interact electrostatically with anionic surface components of both Gram-negative and Gram-positive bacteria, and shown to have the ability to restrict and neutralize microbial infections and to boost additional immune response mechanisms (Skarnes and Watson 1957). In the following decades, AMPs were also identified in plants, insects and vertebrate animals (Cederlund *et al.* 2011).

AMPs are produced by a wide variety of organisms across all domains of life, from bacteria to humans (Zasloff 2002, Yang *et al.* 2014), and constitute what is probably the oldest branch of immunity effectors. Since the identification of lysozyme in 1922, the total number of natural antimicrobial peptides/proteins has expanded vastly to over 2,500, according to the March 2015 update of the Antimicrobial Peptide Database (http://aps.unmc.edu/AP) (Wang *et al.* 2009). This growth has also been driven by the enormous interest in the potential therapeutic applicability of novel AMPs, particularly in the treatment of bacterial pathogens with increasing resistance to traditional antibiotics (Hancock and Sahl 2006).

This section presents the main classes of AMPs, describing their structural and biochemical properties, as well as the most common mechanisms of action. In addition, strategies developed by bacteria to resist against the activity of AMPs are also addressed.

C.1. General features and properties

Despite the source and sequence diversity of the many known natural AMPs (Wang *et al.* 2009), they contain conserved characteristics that are important for their antimicrobial activity: size, charge, amphipathicity and structural conformation (Brogden 2005, Cederlund *et al.* 2011).

AMPs have an average length of 30 amino acid residues (Wang *et al.* 2009, Cederlund *et al.* 2011), which corresponds to an average molecular weight of <5 kDa. The smallest known AMPs, called gageotetrins, are di- and tetrapeptide lipoproteins produced by a marine *B. subtilis* strain (Tareq *et al.* 2014). With 99 and 130 residues, respectively – long enough to be considered small proteins – human lysozyme and β_2 microglobulin are among the longest polypeptides with antimicrobial activity (Wang *et al.* 2009).

A prevalence of basic (arginine, histidine and lysine) over acidic (glutamate and aspartate) amino acid residues confers an overall positive charge to AMPs (+2 to +9; average +3) (Cederlund *et al.* 2011). This cationic character is important for the interaction with surface of bacteria, which has a typical net negative charge conferred by anionic components, such as the Gram-negative lipopolysaccharide (LPS) or the Gram-positive teichoic acids. Nevertheless, some AMPs can also be anionic (Brogden 2005) and, in this case, their interaction with bacterial surfaces appears to be mediated by cationic salt bridges (Harris *et al.* 2009).

Another feature of AMPs is their hydrophobicity: in average, they contain 40–50% of hydrophobic residues (Cederlund *et al.* 2011). These hydrophobic residues are usually distributed through the AMP sequence to promote amphipathicity, i.e. polar and apolar residues are spatially constricted to different sides of the AMP secondary structure. This characteristic is important for solubility in aqueous environments and for AMP activity, which requires integration into membrane lipid bilayers (Lohner *et al.* 2001, Brogden 2005, Ramadurai *et al.* 2010).

AMPs exist in different structural conformations (Fig. 12): (i) linear α-helices, (ii) packed β-sheets, stabilized by internal disulfide bonds; or (iii) extended unorganized structures enriched in specific residues (e.g. proline, arginine or tryptophan) (Brogden 2005, Wiesner and Vilcinskas 2010). The first two structures are the most common in nature (Giuliani *et al.* 2007, Cederlund *et al.* 2011).

Many alpha-helical AMPs are unstructured in aqueous environment and only acquire their final conformation upon interaction with target membranes. This characteristic appears important to prevent AMP cytotoxicity in eukaryotic cells (Nguyen *et al.* 2011). Bend-inducing residues and/or polar sidechains in the hydrophobic face of an alpha-helical AMP create an “imperfect amphipathicity” that favors an efficient membrane insertion and disruption (Mihajlovic and Lazaridis

2010, Nguyen *et al.* 2011). Although intramolecular disulfide bonds are a characteristic feature in β-sheet AMPs and help to stabilize this secondary structure and protect it from proteolytic degradation, they are not essential for antimicrobial activity (Kluver *et al.* 2005, Ramamoorthy *et al.* 2006).

In unstructured extended AMPs, the preponderance of specific amino acids such as tryptophan and arginine is critical in many aspects. The positive charges of arginine attract the peptide towards the anionic bacterial surface, while tryptophan residues not only stabilize the peptide in solution via intramolecular hydrophobic interactions, but promote a strong association to the interfacial region of lipid bilayers (Yau *et al.* 1998, Chan *et al.* 2006). Furthermore, an energetically favorable stacked interaction between the sidechains of both amino acids allows arginine residues to be masked by tryptophan and penetrate the strong apolar membrane environment (Yau *et al.* 1998). Many extended AMPs exert their antimicrobial function not at the membrane level but targeting intracellular components (e.g. proteins and nucleic acids) (Nguyen *et al.* 2011).

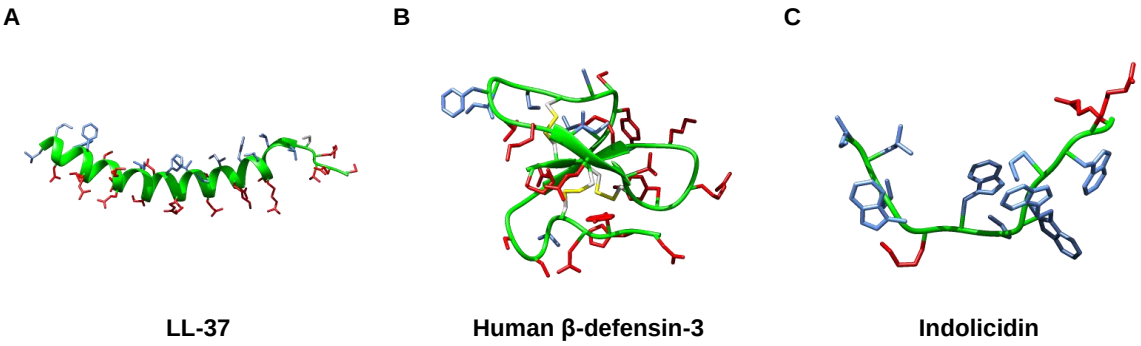

Fig. 12. Main structural conformations of AMPs. (A) Linear α-helices (e.g. LL-37), **(B)** β-sheets (e.g. human β-defensin-3), and **(C)** extended disordered peptides (e.g. indolicidin). Peptide backbone (ribbon) is shown in green, hydrophobic side chains in light blue, polar side chains in red, and disulfide bridges in yellow. Notice the bilateral segregation of hydrophobic and polar residues in LL-37 (important for membrane integration) and the multiple tryptophan sidechains in indolicidin. (PDB IDs: LL-37, 2K6O; human β-defensin-3, 1KJ5; indolicidin, 1G89)

C.2. Classes

AMPs can be classified based in numerous criteria: biosynthesis pathway, biological source, activity, biochemical properties, secondary structure, internal bonding profile and cellular targets (Wang *et al.* 2009).

The simplest classification is based on whether AMPs are generated by ribosomal translation of gene-encoded transcripts or by a ribosome-independent multienzymatic pathway (Wiesner and Vilcinskas 2010). Many bacterial and fungal peptide antibiotics (e.g. bacitracin, vancomycin and polymyxin B) are synthesized through the latter pathway and often possess unusually configurated and non-proteinogenic amino acid residues that can be further modified (Hancock and Chapple 1999). Ribosomally synthesized AMPs are produced by both bacteria – where they take the name of bacteriocins – and eukaryotes.

C.2.1. Bacteriocins

Bacteriocins comprise not only bacterial peptides but also proteins with antimicrobial activity, although it is mostly used in reference to the former group (Cotter *et al.* 2005). These are highly heterogeneous molecules that are produced in response to environmental stresses, such as nutritional shortage and lack of space, to provide a competitive advantage over other microbes. Their variable spectra of activity enable producing bacteria to kill within (narrow spectrum) or outside (broad spectrum) of their species. Importantly, producers contain mechanisms that confer immunity to their own bacteriocins (Cotter *et al.* 2005).

A large majority of the currently known bacteriocins are produced by Gram-positive species, notably Lactobacillales (or lactic acid bacteria, LAB), which include various genera such as *Lactococcus*, *Lactobacillus, Enterococcus* and *Streptococcus* (Cotter *et al.* 2005, Hammami *et al.* 2010). It is not surprising that the growth of research in the area of bacteriocins has been fostered by the food preservation and clinical industries (Cotter *et al.* 2005, Cotter *et al.* 2013, Yang *et al.* 2014). Nonetheless, bacteriocins from Gram-negative bacteria (mainly *E. coli*) and even in Archaea have also been identified and characterized (Hammami *et al.* 2010).

C.2.1.1. Gram-negative bacteriocins

Gram-negative bacteriocins are divided into two groups: colicins and microcins (Table 4) (Yang *et al.* 2014). The first group includes large proteins (25–

Table 4. Examples of Gram-negative bacteriocins.[a]

Bacteriocin	Source	MW[b]	Activity
Colicins			
B	*Escherichia coli*	54,742	Pore formation
E2	*Escherichia coli*	61,561	DNase
E3	*Escherichia coli*	57,960	16S rRNase
E5	*Escherichia coli*	58,254	tRNase
M	*Escherichia coli*	29,453	Peptidoglycan hydrolase
Microcins			
Class I (<5 kDa, PTMs)			
B17	*Escherichia coli*	3,094	DNA gyrase inhibition
J25	*Escherichia coli*	2,107	RNA polymerase inhibition
Class IIa (5–10 kDa, absence of PTMs)			
L	*Escherichia coli*	8,884	Disruption of the inner membrane integrity
Class IIb (5–10 kDa, linear, potential C-terminal PTMs)			
E492	*Escherichia coli, Klebsiella pneumoniae*	7,886	Disruption of the inner membrane integrity

a) Data compiled from: Yang *et al*. 2014, Duquesne *et al.* 2007, Morin *et al.* 2011; Bieler *et al.* 2006.
b) Molecular weight (values in Daltons)

80 kDa) generally encoded in a plasmid-borne gene cluster (Cascales *et al.* 2007). Besides the colicin, the cluster encodes a self-immunity protein, required to protect the producer cell, and a lysis protein, to enable colicin export (van der Wal *et al.* 1995, Cascales *et al.* 2007). Colicins contain three functional domains: a central receptor-binding domain, which recognizes and binds to a target cell surface receptor (Ton or Tol system proteins (Cramer *et al.* 1995)); an N-terminal translocation domain, to transport the surface-bound colicin across the OM; and a C-terminal catalytic domain that can exhibit (i) peptidoglycan hydrolase, (ii) membrane pore-forming or (iii) nuclease activities (Cascales *et al.* 2007).

Contrary to colicins, microcins are significantly smaller polypeptides (<10 kDa, hence the prefix “micro”) and may present post-translational modifications (PTMs), such as adenyl groups, thiazole/oxazole rings and lasso-type cyclization. They are heat- and pH-stable, resistant to proteolytic digestion and exhibit a highly potent activity (nM range) against a small subset or targets (Duquesne and Destoumieux-Garzón 2007). Like colicins, they are encoded in large gene clusters present in plasmids (and also in the chromosome), and expressed as immature precursors (promicrocins) with cleavable N-terminal signal peptides. Together with the genes encoding the promicrocin and the self-immunity protein(s), further genes coding for an ABC transporter-based export system and PTM enzymes are also included (Duquesne and Destoumieux-Garzón 2007).

Microcins are classified into two classes: class I, including low-molecular weight peptides (<5 kDa) with extensive PTMs; and class II, comprising heavier peptides (5–10 kDa). The latter group is subdivided into classes IIa (no PTMs) and IIb (chromosome-encoded linear peptides with potential C-terminal PTMs). Functionally, microcins were found to target the cytoplasmic (or inner) membrane as well as the intracellular enzymatic complexes required for nucleic acid synthesis (Duquesne and Destoumieux-Garzón 2007).

C.2.1.2. Gram-positive bacteriocins

The majority of Gram-positive bacteriocins are biochemically similar to Gram-negative microcins, in that they are also low-molecular weight (<10 kDa), heat-stable peptides that may contain PTMs, although not as extreme as the ones present in microcins. They are also encoded in a cluster with a self-immunity gene and are exported via an ABC transporter system (Duquesne and Destoumieux-Garzón 2007).

These Gram-positive AMPs are distributed between two classes (Table 5). Historically, a third class encompassed large-sized antimicrobial proteins called bacteriolysins (e.g. lysostaphin and enterolysin A) (Schindler and Schuhardt 1964, Nilsen *et al.* 2003), which were similar in structure and activity to colicins. However, they are no longer considered bacteriocins and this class was constituted as a separate group (Cotter *et al.* 2005).

Class I (lantibiotics)

Class I bacteriocins consist of small peptides (<5 kDa) featuring PTMs such as dehydrated (dehydroalanine and dehydrobutyrine) and/or thiother-containing (lanthionine and β-methyllanthionine) amino acids (Islam *et al.* 2012). The unusual lanthionine amino acid – for which members of this class are also called lantibiotics (lanthionine-containing antibiotics) – results from a thioether bond between the sidechains of cysteine and dehydrated residues. This intramolecular link generates the ring or loop structures that are typical of lantibiotics (Fig. 13) (Cotter *et al.* 2005). Depending on the number and location of these lanthionine

bridges, lantibiotics can have linear (type A) or globular (tybe B) conformations (Jung 1991). While type-A peptides are cationic, type-B are usually neutral or anionic (Islam *et al.* 2012). A third type includes lantibiotics comprised of two peptides acting synergistically, an example of which is lacticin 3147 (Fig. 13) (Lawton *et al.* 2007).

The antimicrobial activity of lantibiotics is exerted through (i) inhibition of cell wall biosynthesis and/or (ii) formation of membrane pores (Islam *et al.* 2012). Nisin A, the best known lantibiotic and first bacteriocin to be identified (Rogers and Whittier 1928), is a bifunctional type-A peptide that kills Gram-positive bacteria by inhibiting peptidoglycan synthesis, through its binding to lipid II, and making pores in the membrane (Fig. 14) (Brotz *et al.* 1998b, Wiedemann *et al.* 2001). This dual role is encoded in its bipartite structure, whereby the binding of the N-terminus end of nisin to lipid II pyrophosphate (Hsu *et al.* 2004) positions the flexible C-terminus end for membrane insertion (van Heusden *et al.* 2002). Mersacidin, a type-B lantibiotic is only able to interact with lipid II to disrupt cell wall synthesis (Brotz *et al.* 1998b). Two-peptide lantibiotics seem to have receptor-binding and pore-forming activities allocated to different peptides (Martin *et al.* 2004).

Another lantibiotic receptor is phosphatidylethanolamine (PE), a membrane phospholipid. By binding to PE, cinnamycin and duramycins inhibit phospholipase A2 activity (Fredenhagen *et al.* 1990). Interestingly, lantibiotics like the enterococcal cytolysin can also act as virulence factors against mammalian cells (Van Tyne *et al.* 2013).

Class II (non-lantibiotics)

The members of this class are also small (<10 kDa) and heat-stable peptides, but unlike lantibiotics they do not contain lanthionine residues or other complex PTMs (Cotter *et al.* 2005). They are highly potent AMPs (nM range) that function by disrupting the membrane (Nissen-Meyer *et al.* 2009).

Four subclasses accommodate class II bacteriocins according to structure and sequence similarity (Cotter *et al.* 2005): (i) class IIa, pediocin-like or *Listeria*-active peptides; (ii) class IIb, two-peptide peptides; (iii) class IIc, cyclic peptides; and (iv) class IId, linear non-pediocin-like single peptides.

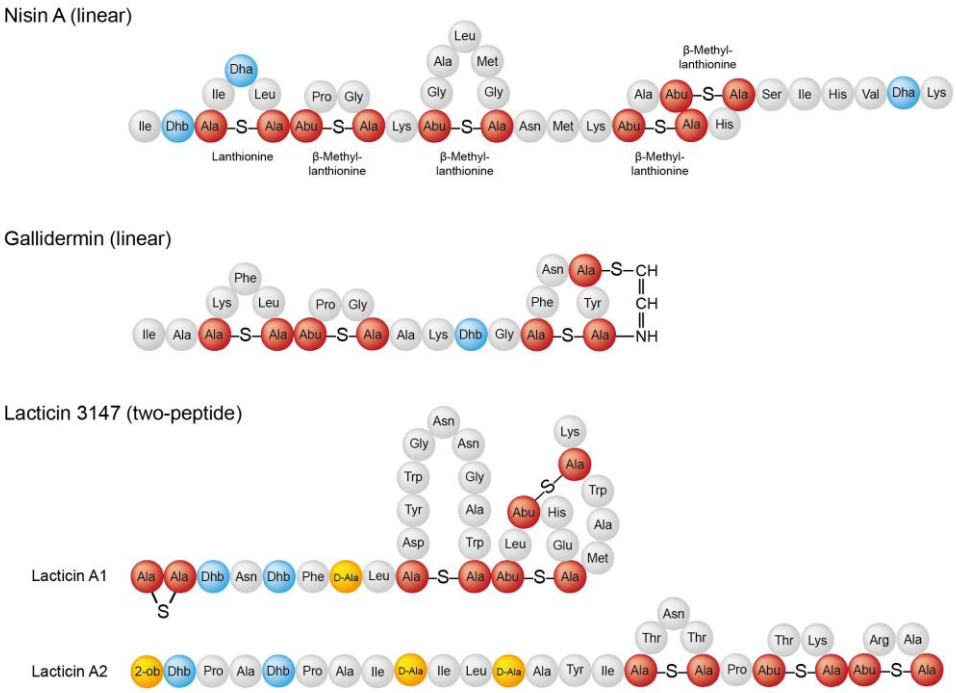

Fig. 13. Schematic representation of the structure of the lantibiotics nisin A, gallidermin and lacticin 3147. These Gram-positive bacteriocins possess characteristic lanthionine residues (red) that are formed when a thioether linkage is created between the sulfhydril group of a cysteine and dehydrated residues (blue), such as dehydroalanine (Dha) and dehydrobutyrine (Dhb). When Dha is involved, a lanthionine bridge (Ala-S-Ala) is formed, whereas a β-methyl-lanthione bond (Abu-S-Ala) is created when Dhb is the acceptor residue. Other unusual amino acids (yellow), such as D-alanine (D-Ala) or 2-oxobutyrate (2-ob) might also be present. Abu, aminobutyrate. (Adapted from Cotter *et al.* 2005 and Kellner *et al.* 1988)

Class IIa (pediocin-like). This designation originates from one of the first identified members of this subclass (pediocin PA-1), which currently contains over 20 peptides and is probably the most well-characterized due to their high antimicrobial specificity towards *Listeria* (Eijsink *et al.* 1998). These peptides range between 38 (e.g. leucocin A, mesentericin Y105) and 47 (e.g. carnobacteriocin B2) residues and contain an N-terminal region with a highly conserved YGNG(V/L)XC(X)$_4$CXV sequence ("pediocin box") and a less conserved hydrophobic C-terminal region (Nissen-Meyer *et al.* 2009). Structural studies revealed that the cationic N-terminal region forms a disulfide bond-stabilized β-sheet structure that sits at the membrane interface, while the C-terminal domain is folded into a hydrophobic hairpin structure (Fregeau Gallagher *et al.* 1997) that buries into the apolar core of the membrane bilayer. The structure and sequence variability of the C-terminal hairpin play an important role in determining target cell specificity (Johnsen *et al.* 2005), by recognizing the mannose-specific

phosphotransferase system (Man-PTS) permease (Fig. 14) (Ramnath *et al.* 2000, Dalet *et al.* 2001, Hechard *et al.* 2001, Diep *et al.* 2007), similarly to microcin E492 (Duquesne and Destoumieux-Garzón 2007). In this case, the corresponding self-immunity factors counteract pediocin-like activity by interfering with their recognition of the mannose permease (Johnsen *et al.* 2005).

Class IIb (two-peptide). This subclass contains bacteriocins that are only active when two related non-active peptides come together (Moll *et al.* 1996), similar to two-peptide lantibiotics (Lawton *et al.* 2007). Lactococcin G from *Lactococcus lactis* was the first of currently over 15 class IIb bacteriocins to be identified (Nissen-Meyer *et al.* 2009). Other examples include enterocin 1071, lactacin F, ABP-118 and various plantaricins (Nissen-Meyer *et al.* 2009). Unstructured while separate in solution (Hauge *et al.* 1998), the two peptides fold into alpha-helical conformations when they interact with each other and insert into membranes. Recently, their receptor was revealed to be UppP, an integral membrane protein that regenerates bactoprenol-phosphate for peptidoglycan and WTA biosynthesis (Fig. 14) (Kjos *et al.* 2014). This inter-peptide interaction is promoted and stabilized by GXXXG motifs present in both peptides (Rogne *et al.* 2008). In the membrane, the two-peptide bacteriocin forms a selective ion-permeable pore that dissipates the proton-motive force and lead to cell death (Nissen-Meyer *et al.* 2009). Mutagenesis studies demonstrated that the target specificity region of these bacteriocins was located to the β peptide N-terminus (Oppegard *et al.* 2007), and that the self-immunity protein recognizes the bacteriocin helix-helix structure (Nissen-Meyer *et al.* 2009).

Class IIc (cyclic). In the last step of biosynthesis, peptides from this subclass of bacteriocins undergo cyclization by covalent linkage of their N- and C-termini, through a yet unclear enzymatic mechanism (Maqueda *et al.* 2008). Apart from subtilosin A (Babasaki *et al.* 1985, Kawulka *et al.* 2003), all cyclic bacteriocins are positively charged peptides (Nissen-Meyer *et al.* 2009). Their mode of action is similar to class IIb bacteriocins and cyclization appears to promote overall structure stabilization and increased resistance to proteolysis (Maqueda *et al.* 2008). The first identified and best-studied cyclic bacteriocin is enterocin AS-48

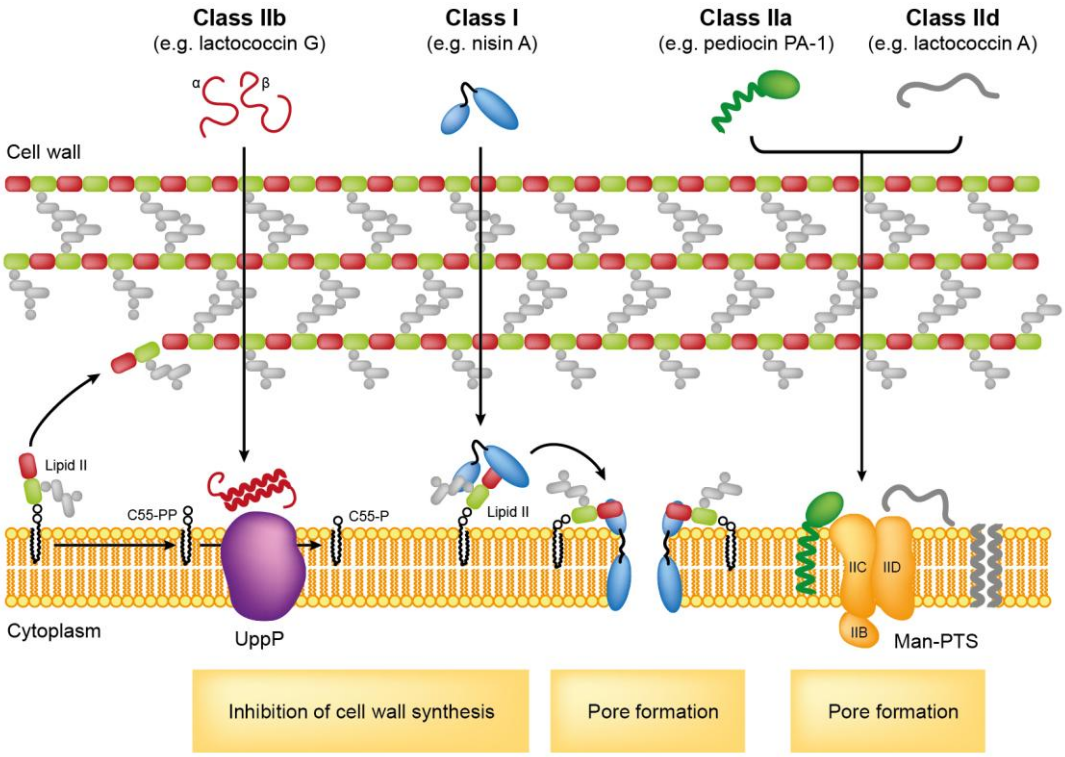

Fig. 14. Mechanisms of action of representative Gram-positive bacteriocins. Bacteriocins can promote bacterial death by disrupting the cell wall biosynthesis or by directly creating pores in the cytoplasmic membrane, both mechanisms leading to cell lysis. Lantibiotics (class I) bind to and hijack the peptidoglycan precursor lipid II, while class IIb peptides have been shown to bind to UppP, a bactoprenol-phosphate (C55-P) recycling membrane protein. Some of these receptor-bound AMPs, like nisin A, can additionally interact with the lipid bilayer and induce pore formation. Other class II members (e.g. pediocins and lactococcin A) are also able to disrupt the membrane integrity, after binding with high specificity to Man-PTS membrane proteins. (Adapted from Cotter *et al.* 2013).

(Galvez *et al.* 1986, Martinez-Bueno *et al.* 1994), an alpha-helical globular peptide with broad antimicrobial spectrum. Membrane insertion requires structural transition from a water-soluble to a membrane-bound conformation, which exposes hydrophobic helices that penetrate the membrane (Maqueda *et al.* 2008).

Class IId (single, linear, non-pediocin-like). In this subclass are included single, linear, unmodified peptides with no sequence similarity with pediocin-like bacteriocins (Cotter *et al.* 2005). Its first member, lactococcin A, was isolated in 1991 from *L. lactis* and shown to have a narrow antimicrobial spectrum (Holo *et al.* 1991, van Belkum *et al.* 1991). Interestingly, its mechanism of action is similar to class IIa peptides: binding to the Man-PTS permease to induce membrane permeabilization (Fig. 14). Lactococcin A inhibition by self-immunity proteins works

Table 5. Examples of Gram-positive bacteriocins.[a]

Bacteriocin	Producing strains	MW[b]	Activity	References
Class I (lantibiotics)				
Type A (linear)				
Nisin A	*Lactococcus lactis*	3,352	Cell wall synthesis inhibition Membrane pore formation	Brotz *et al.* 1998b; Wiedemann *et al.* 2001
Epidermin	*Staphylococcus epidermidis* Tü 3298, 1580	2,164	Cell wall synthesis inhibition	Allgaier *et al.* 1986; Götz *et al.* 2014
Gallidermin	*Staphylococcus gallinarum* Tü 3298	2,164	Cell wall synthesis inhibition	Kellner *et al.* 1988; Götz *et al.* 2014
Type B (globular)				
Mersacidin	*Bacillus* sp. HIL-Y85/54728	1,824	Cell wall synthesis inhibition	Chatterjee *et al.* 1992; Brotz *et al.* 1998a
Two-peptide				
Lacticin 3147	*Lactococcus lactis* DPC3147	3,449 (A1)[c] 3,006 (A2)[c]	Cell wall synthesis inhibition Membrane pore formation	Ryan *et al.* 1996; McAuliffe *et al.* 1998
Class II (non-lantibiotics)				
Class IIa (pediocin-like)				
Pediocin PA-1	*Pediococcus acidilactici* PAC-1.0	4,629	Membrane disruption	Henderson *et al.* 1992
Class IIb (two-peptide)				
Lactococcin G	*Lactococcus lactis* LMGT2081	4,346 (α) 4,110 (β)	Membrane disruption	Nissen-Meyer *et al.* 1992; Moll *et al.* 1996
Class IIc (cyclic)				
Enterocin AS-48	*Enterococcus faecalis* S-48	7,149	Membrane disruption	Gálvez *et al.* 1986; Martinez-Bueno *et al.* 1994
Class IId (single, linear, non-pediocin-like)				
Lactococcin A	*Lactococcus lactis* ssp. *cremoris* LMG2130	5,778	Membrane disruption	Holo *et al.* 1991

a) Data compiled from: Yang *et al*. 2014, Cotter *et al.* 2005, Nissen-Meyer *et al.* 2009, Wang *et al.* 2009.
b) Molecular weight (values in Daltons).
c) Values obtained from BACTIBASE (Hammami *et al.* 2010).

by preventing this interaction (Diep *et al.* 2007). Staphylococcal) class IId bacteriocins have been identified (e.g. aureocins A53 and A70).

C.2.2. Defensins

Defensins were first identified in rabbit peritoneal neutrophil granulocytes as small cationic peptides with broad-spectrum antimicrobial activity (Selsted *et al.* 1984). Soon after, similar peptides were also isolated from human neutrophils

(Ganz *et al.* 1985) and detected also in epithelial cells (Ouellette *et al.* 1989, Diamond *et al.* 1991). Nowadays, defensins are known to be present not only in mammals but also in birds, reptiles, invertebrates, as well as in plants and fungi, presenting themselves as one the oldest and most conserved AMP families (Wong *et al.* 2007).

Elucidation of the structure and activity of defensins was obtained with the extensive study and characterization of the peptides from mammalian origin. Regardless of their biological source, defensins have two defining features: (i) an anti-parallel β-sheet fold and (ii) six conserved cysteine residues that pair up covalently through three intramolecular disulfide bridges and help stabilizing the peptide structure (Ganz 2003).

According to the relative position of the cysteines within the peptide sequence and the cysteine pairs linked by disulfide bonds, defensins can be distributed into alpha (α)- and beta (β)-defensins. While in α-defensins, two of the three disulfide bonds occur between C1–C6 and C3–C5, in β-defensins they occur between C1–C5 and C3–C6 (Fig. 14). However, this mismatch does not translate into significant conformational differences between both groups (Zimmermann *et al.* 1995). Moreover, the proximal chromosomal localization of genes for both defensin families indicates that they evolved from a common ancestral defensin (Liu *et al.* 2007) and diverged with rodents and primates, where α-defensins are exclusively synthesized (Patil *et al.* 2004). A third group of α-defensin-derived cyclic peptides were identified in rhesus macaque leukocytes. Their unusual structure and molecular origin puts these defensins into another sub-family: the theta (θ)-defensins (Fig. 15) (Tang *et al.* 1999). They have evolved in simians and are still found in Old World monkeys but mutations led to their inactivation in humans and other hominid primates (Cole *et al.* 2002, Nguyen *et al.* 2003). Contrasting with other defensins, θ-defensins are significantly better antiviral effectors (Munk *et al.* 2003).

Both α- and β-defensins are generated by successive proteolytic cleavages of larger (up to 100 aa) inactive precursors called preprodefensins (Harwig *et al.* 1992, Valore and Ganz 1992). These contain an N-terminal leader peptide (~19 aa, pre-sequence) and the C-terminal mature defensin (15–45 aa) (Ganz 2003). The precursor of α- and θ-defensins, but not of β-defensins, contains a large

central pro-domain (~40 aa) with acidic nature. This pro-piece seems to balance the positive charge of the C-terminal defensin region within the overall propeptide, and inhibit undesirable cytotoxic effects of the mature peptide within the producer cell (Michaelson *et al.* 1992, Valore *et al.* 1996). As for θ-defensins, they arise from the head-to-tail cyclization of two nonapeptide fragments excised from larger α-defensin paralog precursors (Fig. 15) (Tang *et al.* 1999).

Unsurprisingly, defensins are highly abundant in phagocytes and epithelia (Table 6), which constitute primary sites of host interaction with microorganisms. In particular, subcellular granules like those present in neutrophils and Paneth cells –

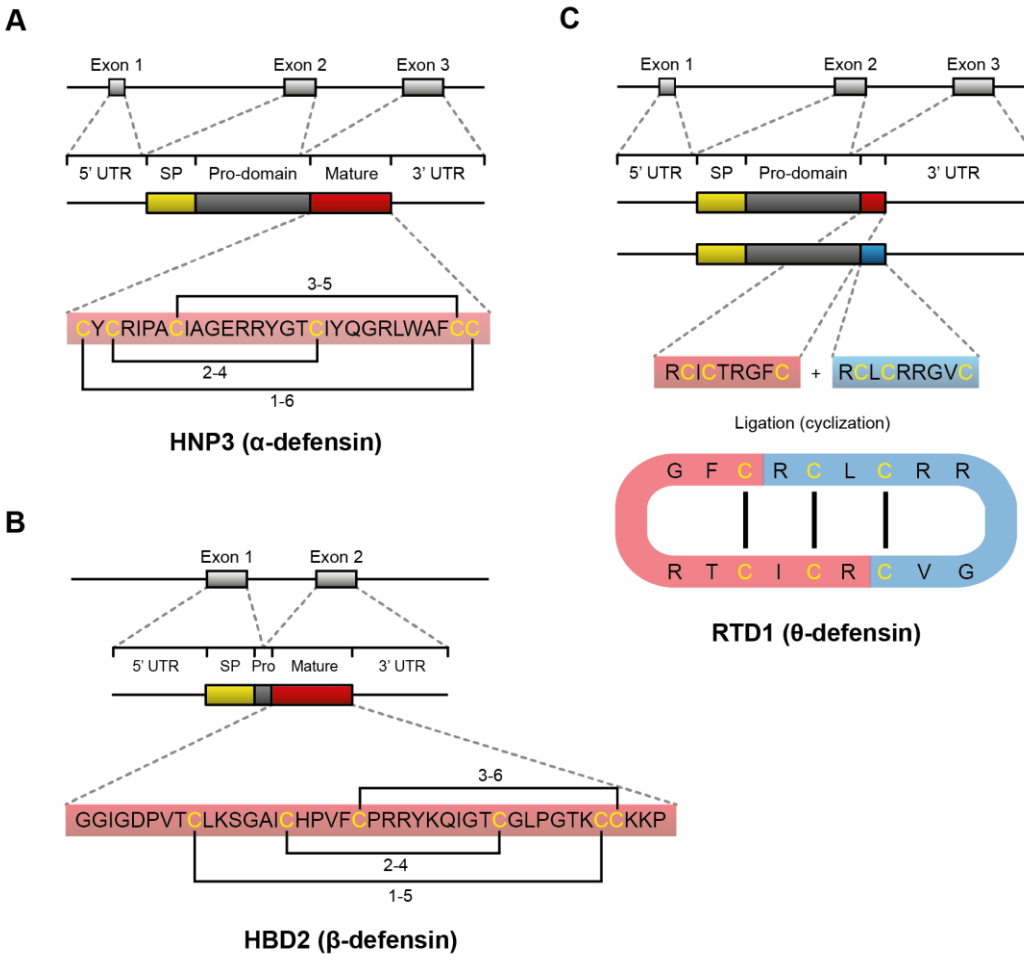

Fig. 15. Genetic organization and protein processing of defensins. Defensin peptides are initially expressed as part of a larger precursor (preprodefensin), containing an N-terminal signal peptide (SP), a central pro-domain, and the mature bioactive peptide in the C-terminus. Following cleavage of the C-terminal peptide, three intramolecular disulfide bonds are established between the six conserved cysteines. In **(A)** α-defensins, pairing occurs with C1-C6, C2-C4 and C3-C5, while in **(B)** β-defensins, it involves C1-C5, C2-C4 and C3-C6. The biogenesis of **(C)** θ-defensins requires the ligation/cyclization of two mature nonapeptides, each with three cysteines. HNP3, human neutrophil peptide 3; HBD2, human beta-defensin 2; RTD1, rhesus theta-defensin 1; UTR, untranslated region. (Adapted from Ganz 2003, Selsted and Ouellette 2005).

Table 6. Examples of mammalian defensins.[a,b]

Defensins	Cell/tissue source	Synthesis	Release[c]
Alpha (α)-defensins			
HNP1–4 (human)	Granulocytes, monocytes, lymphocytes	Constitutive, inducible (pro-inflammatory cytokines, NOD2 agonists)	Degranulation (phagocytosis)
HD5–6 (human)	Paneth cells, urogenital tissue cells	Constitutive, inducible (NOD2 agonists)	Degranulation (bacterial antigens, cholinergic agonists)
Cryptdins (mouse)	Paneth cells	Constitutive, inducible (NOD2 agonists)	Degranulation (bacterial antigens, cholinergic agonists)
Beta (β)-defensins			
HBD1 (human)	Epithelial cells (e.g. keratinocytes), monocytes	Constitutive, inducible (LPS, IFN-γ)	Secretion
HBD2–3 (human)	Epithelial cells (e.g. keratinocytes), monocytes, DCs	Inducible (pro-inflammatory cytokines, TLR and NOD agonists)	Secretion
HBD4 (human)	Epithelial cells (testis, epididymis)	Inducible (bacteria, PMA)	Secretion
TAP (bovine)	Trachea	Inducible (bacteria, LPS)	Secretion
Theta (θ)-defensins			
RTD1 (rhesus macaque)	Neutrophils, Paneth cells	Constitutive, inducible (virus)	Degranulation (phagocytosis) Secretion

a) Data compiled from: Hazlett and Wu 2011, Ganz 2003, Diamond *et al.* 2000, Kaiser and Diamond 2000, Tang *et al.* 1999 and Lucero *et al.* 2013.
b) DCs, dendriti cells; IFN-γ, interferon-gamma; NOD2, nucleotide-binding oligomerization domain (NOD)-containing protein 2; PMA, phorbol myristate acetate; TAP, tracheal antimicrobial peptide.
c) Degranulation-inducing stimuli are listed between parentheses.

epithelial cells located at the bottom of intestinal crypts, a highly sterile niche due to the abundance of AMPs secreted by these cells – contain the highest concentration of defensins, in the mM range (Ganz 1987, Ayabe *et al.* 2000).

Defensin synthesis and secretion can be constitutive or triggered by local pro-inflammatory or bacterial stimuli (Table 6) (Ganz 2003). In granulocytes, synthesis, Golgi maturation and vesicular storage of α-defensins concur with granulopoiesis in the bone marrow (Yount *et al.* 1995). After phagocytosis, these granules fuse with phagosomes, releasing the mature defensin peptides onto the ingested microorganism(s). Intestinal α-defensins such as mouse cryptdins (i.e. crypt defensins) and the human defensin-5 (HD5) are also constitutively synthesized. However, they are processed into their bioactive forms in different ways: whereas cryptdins are activated by the matrix metalloproteinase 7 (MMP-7, or matrilysin) and stored as bioactive peptides in Paneth cell secretory granules (Wilson *et al.* 1999), the HD5 propeptide is cleaved by a trypsin isoform only

during or after secretion (Ghosh *et al.* 2002). In any case, Paneth cell degranulation into the crypts occurs quickly in response to the sensing of bacteria or bacterial antigens (Ayabe *et al.* 2000). Other epithelial defensins, such as the human β-defensins 2 (HBD2), HBD3 and HBD4 and the bovine tracheal β-defensin, are transcriptionally induced by cytokines or microbial factors, which activate signaling pathways mediated by NF-κB or other transcription factors (Table 6) (Diamond *et al.* 2000, Hertz *et al.* 2003, Liu *et al.* 2003, Sorensen *et al.* 2003, Proud *et al.* 2004).

Besides their main and direct antimicrobial activity, defensins have also been found to play an important role in the inflammatory response by modulating the production of pro-inflammatory cytokines and chemokines (Nagaoka *et al.* 2008, Miles *et al.* 2009). In addition, defensins can also behave as chemoattractant factors for various cellular players of the innate and adaptive immune responses and stimulate angiogenesis (Yang *et al.* 1999, Yang *et al.* 2000, Chavakis *et al.* 2004, Rohrl *et al.* 2010). Unlike their antimicrobial activity, the chemotactic properties of defensins are highly dependent on their structure (Wu *et al.* 2003).

C.2.3. Cathelicidins

The cathelicidins are another well-characterized family of AMPs, and together with defensins, the most important class of mammalian AMPs. However, unlike defensins, they are not as evolutionarily conserved and widespread in nature, with all its currently known members having been identified in vertebrates, including humans, murines and several domesticated even-toed ungulates (Table 7) All cathelicidin-expressing species have multiple genes for these AMPs, except humans, monkeys, murines, rabbits and guinea pigs, which have only one (Kościuczuk *et al.* 2012). Also contrasting with the defensin family is the high heterogeneity verified among mature cathelicidin peptides, which can range in length between 12 and 100 residues and present all kinds of structures (Zanetti *et al.* 1995). Indeed, cathelicidins can adopt the more common linear α-helical (e.g. human LL-37, rabbit CAP-18 and mouse CRAMP), a disulfide-bridged β-stranded (protegrins) or a linear unstructured Pro/Arg/Trp-rich conformation (e.g. porcine PR-39 and bovine bactenecins and indolicidin) (Gennaro and Zanetti 2000).

Despite their significant structural diversity, an element common to all cathelicidin peptides and that constitutes the hallmark of this family resides in the N-terminal domain of their unprocessed precursors (Zanetti *et al.* 1995). Whereas the C-terminal region of cathelicidin prepropeptides shows high sequence variability, consistent with the diversity observed in the mature peptides, the N-terminal preproregion is very much conserved. Linking the signal peptide (~30 aa) at the N-terminus to the C-terminal bioactive peptide is a propiece (99–114 aa) with 70% homology to a cysteine proteinase inhibitor protein, called cathelin (for cathepsin L inhibitor) (Zanetti *et al.* 1995), found in pig leukocytes (Fig. 16) (Ritonja *et al.* 1989). It is the presence of this cathelin-like prodomain that lends its name to the cathelicidin family (Zanetti *et al.* 1995) and which enabled that a Bac5, a bactenecin previously isolated from bovine neutrophils (Gennaro *et al.* 1989) was identified as the first cathelicidin member (Zanetti *et al.* 1993). Cathelicidin precursors were first detected in neutrophil cells, thus cathelicidins are alternatively named myeloid antimicrobial peptides (MAP) and some mature peptides carry the acronym in the name (e.g. porcine PMAP-23 or sheep SMAP-29) (Kościuczuk *et al.* 2012).

Cathelicidin precursors are transcribed from four-exon genes, where the first three exons encode the N-terminal preproregion and the more variable exon 4 encodes the mature AMP-containing domain (Fig. 16). Binding sites for hematopoietic and pro-inflammatory transcription factors have been identified in some promoters, indicating potentially inducible cathelicidin expression (Gudmundsson *et al.* 1995, Zhao *et al.* 1995, Larrick *et al.* 1996). Like defensins, neutrophil cathelicidins are expressed and processed in myeloid precursor cells in the bone marrow, accumulating as inactive preforms in cytoplasmic granules (Zanetti *et al.* 1990, Sorensen *et al.* 1997). The lack of antimicrobial activity of the propeptide appears to result from an inhibitory action of the cathelin domain, whose anionic nature cancels the cationicity of the C-terminal AMP domain (Scocchi *et al.* 1992). Upon proper stimulation, the propeptides are released by degranulation and processed into their final bioactive form following a neutrophil elastase-mediated proteolytic cleavage of a consensus sequence located at the end of the cathelin domain (Zanetti *et al.* 1991, Zanetti *et al.* 1995, Panyutich *et al.* 1997). However, not every cathelicidin precursor is fully processed (Sorensen *et*

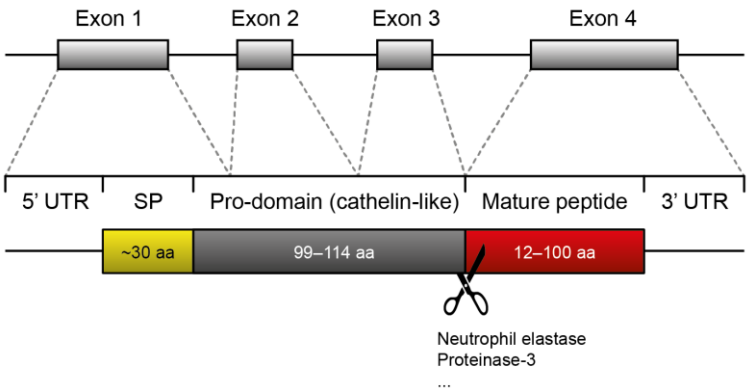

Fig. 16. Structural organization of cathelicidin genes and protein precursors. Cathelicidin peptides are expressed as part of a larger precursor encoded by four different exons, of which the last encodes specifically the mature peptide. The cathelicidin precursor contains an N-terminal signal peptide (SP), a central pro-domain highly homologous to the protease inhibitor cathelin, and the mature bioactive peptide in the C-terminus. The final proteolytic cleavage, which releases the mature peptide, occurs during secretion upon certain stimuli and is mediated by neutrophil elastases or other proteases, such as proteinase-3. UTR, untranslated region. (Adapted from Ramanathan *et al.* 2002).

al. 1999). Conversely, the same propeptide may require different proteases to become active. This is the case of the LL-37 precursor, hCAP-18, which can be cleaved by proteinase-3 in neutrophils (Sorensen *et al.* 2001) or other proteases in other tissues to generate different cleavage products (Sorensen *et al.* 2003, Murakami *et al.* 2004).

Initially believed to be exclusively produced in immature granulocytes, the human and murine cathelicidins were also found to be synthesized by other leukocytes (e.g. monocytes, NK cells) as well as different types of cells (e.g. keratinocytes and epithelial cells of the intestinal, respiratory and urogenital tracts), where their expression is constitutive and/or inducible by microbial, inflammatory or developmental factors (Zanetti 2005).

Due to their cationic and amphipathic properties, cathelicidins exert their antimicrobial activity by targeting and disrupting anionic microbial membranes, through the formation of transmembrane pores (Ramanathan *et al.* 2002). Bovine bactenecins and porcine peptides, however, have been shown to target intracellular components without compromising the membrane (Lee *et al.* 2009). Cathelicidins are active against both Gram-negative and Gram-positive bacteria, although with structure-dependent potency differences. Moreover, some cathelicidins, like LL-37 and the porcine indolicidin and protegrin-1, are able to

Table 7. Examples of mammalian cathelicidins.[a]

Cathelicidin	Origin	Cell/tissue source	Functions
Alpha (α)-helical			
hCAP-18/LL-37	Human	Neutrophils, monocytes, lymphocytes, epithelial cells (keratinocytes, intestinal, respiratory and urogenital mucosae)	Antimicrobial activity (pore formation), LPS inhibition, wound repair, angiogenesis, immune response modulation (leukocyte chemotaxis)
CRAMP	Mouse	Neutrophils, mast cells, spleen, epithelial cells (keratinocytes, gastrointestinal, respiratory and urogenital mucosae)	Antimicrobial activity (pore formation)
CAP-18	Rabbit	Neutrophils	Antimicrobial activity, LPS inhibition
BMAPs	Cattle	Neutrophils, lymphoid organs, tongue, mammary glands, reproductive tract	Antimicrobial activity (pore formation), immune response modulation, tumor cell apoptosis
Beta (β)-sheet			
Protegrins	Pig	Bone marrow, neutrophils, leukocytes	Antimicrobial activity (pore formation), LPS inhibition
Extended Pro/Arg/Trp-rich			
Bactenecins	Cattle	Neutrophils, lymphoid organs	Antimicrobial activity (pore formation, inhibition of cell wall, protein and nucleic acid synthesis)
Indolicidin	Cattle	Neutrophils	Antimicrobial activity (inhibition of nucleic acid synthesis). LPS inhibition, immune response modulation
PR-39	Pig	Bone marrow, neutrophils, lymphoid organs, small intestine	Antimicrobial activity (inhibition of nucleic acid synthesis), immune response stimulation (leukocyte chemotaxis)

a) Data compiled from: Ramanathan *et al.* 2002, Zanetti 2004, Zanetti 2005 and Kosciuczuk *et al.* 2012.

neutralize fungi and enveloped viruses (Ramanathan *et al.* 2002, Kai-Larsen and Agerberth 2008).

Cathelicidin peptides can additionally perform other non-microbicidal functions that contribute to the mounting of a prompt and adequate immune response against microbial pathogens or other biological challenges. For instance, the rabbit and human CAP-18 propeptides and the mature LL-37 and porcine peptides were shown to bind and inactivate LPS, reducing its toxic effects during infection (Bevins 1994, Larrick *et al.* 1994, Larrick *et al.* 1995, Falla *et al.* 1996, Kirikae *et al.* 1998). PR-39 was implicated in tissue protection against excessive inflammation by inhibiting ROS generation via the phagocyte NAPDP oxidase (Shi *et al.* 1996). PR-39 and LL-37 were also detected in wound fluids, where they promote the activation of tissue repair and cell proliferation mechanisms, including angiogenesis (Gallo *et al.* 1994, Vandamme *et al.* 2012). In addition, both AMPs display chemoattractant properties, stimulating leukocyte recruitment to sites of

inflammation (Huang *et al.* 1997, Yang *et al.* 2000). This places cathelicidins, alongside other AMPs, in the molecular bridge connecting the innate and adaptive arms of immunity.

C.3. Mechanisms of action

C.3.1. Cytoplasmic membrane disruption

The main target of AMPs is the microbial membrane, whose disruption ultimately results in cell death. The physicochemical properties of AMPs selected them as ideal bacterial killing effectors, due to their inherent affinity and specificity for prokaryotic cell surface. Indeed, their cationic nature drives an electrostatic interaction with the anionic cell envelope of bacteria, while bypassing the neutrally charged eukaryotic membrane. This net negative charge is not only conferred by secondary surface glycopolymers (e.g. LPS or TAs) but also by acidic membrane phospholipids (Brogden 2005), such as phosphatidylglycerol (PG), phosphatidylserine and cardiolipin (Yeaman and Yount 2003). In contrast, eukaryotic membranes contain neutral phospholipids like PE, phosphatidylcholine and sphingomyelin, and are further supplemented and differentiated with the presence of neutral sterols (Yeaman and Yount 2003). Upon binding to bacterial surfaces, additional parameters such as hydrophobicity, amphipathicity and structural conformation play a key role in AMP interaction with the cytoplasmic membrane. Moreover, the peptide/lipid ratio guides the orientation of AMPs in the membrane: as the first increases, so does the perpendicularity of peptides relative to the bilayer and the propensity for membrane disruption (Lee *et al.* 2004, Brogden 2005).

Depending on their intrinsic properties and on membrane composition and architecture, AMPs can mediate destabilization of the membrane integrity by different mechanisms (Nguyen *et al.* 2011). Three main or classic models are acknowledged: (i) the barrel-stave, (ii) the toroidal-pore, and (iii) the carpet model (Fig. 17) (Yeaman and Yount 2003).

As its name indicates, the barrel-stave mechanism results in the formation of a barrel-like ring transmembrane pore, where each stave corresponds to an AMP

monomer from a larger oligomerized complex (Fig. 17) (Ehrenstein and Lecar 1977). As an increasing number of peptide molecules begin to penetrate the membrane surface, thermodynamically favorable monomer aggregation promotes a transmembrane pore configuration, where internal hydrophobic residues face out towards the apolar membrane core and hydrophilic sidechains line the inner aqueous channel (Breukink and de Kruijff 1999). The fungal α-helical peptide antibiotic alamethicin is one of very few and best-studied AMPs following this type of mechanism (Fox and Richards 1982, Sansom *et al.* 1991, Beven *et al.* 1999).

Comparatively, the toroidal-pore model is observed in a much larger number and diversity of AMPs such as mellitin, magainins, protegrins and LL-37 (Yang *et al.* 2001, Henzler Wildman *et al.* 2003). In this membrane-disruptive mechanism, a transmembrane pore is also formed but differs structurally from the barrel-stave pore in that phospholipids are intercalated with the peptide monomers (Fig. 17). As α-helical peptides bind to the membrane, they push the outer membrane leaflet inwards, forcing a positive curvature strain that promotes further peptide insertion (Hallock *et al.* 2003). Processive AMP oligomerization together with the fusion of both membrane leaflets into a toroid-like surface result in the assembly of a toroid-like pore with a luminal lining composed of the hydrophilic surface of peptide monomers alternated with the polar head groups of phospholipids (Matsuzaki *et al.* 1996, Hara *et al.* 2001, Yang *et al.* 2001). Toroidal pores have less monomers but appear to be wider than barrel-stave pores, which seems to result from the electrostatic stability provided by the alternation of anionic (phospholipid) and cationic (peptide) charges on the channel surface (Yang *et al.* 2001). Further studies on the interaction of mellitin with membranes pointed however that toroidal pores may not be as structurally organized or require that many monomers to be formed (Sengupta *et al.* 2008).

The third model is also the only that does not rely in structured pore formation for membrane disruption. In the “carpet” mechanism, linear α-helical AMPs, such as the invertebrate cecropins (Oren and Shai 1998), or the more globular β-sheet defensins (Ganz 2003) adhere to the outer membrane leaflet in a dispersed fashion – covering it like a carpet – until they reach a threshold concentration which triggers its disintegration in a detergent-like manner, often resulting in formation of micelles (Fig. 17) (Ladokhin and White 2001, Shai and

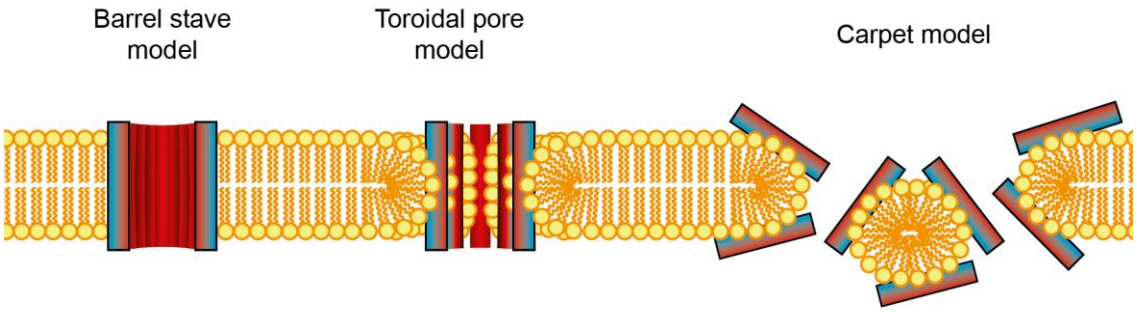

Fig. 17. Main models of AMP-mediated disruption of bacterial cytoplasmic membrane. In the barrel-stave model, a barrel-like ring transmembrane pore is formed, where each stave corresponds to a monomer from a larger oligomerized complex. The hydrophobic side of the peptide (blue) is faced against the apolar membrane core while the hydrophilic side (red) is faced towards the inner aqueous channel. In the toroidal pore model, a similar complex is formed but monomers are intercalated by phospholipid head groups as result of the curvature and fusion of the two membrane leaflets. The carpet model consists in a dispersed micelle-like dissolution of the membrane as the amount of bound peptide reaches a critical threshold. Some AMPs may follow more than one of these models during their interaction with bacterial membranes.

Oren 2001). AMPs like mellitin, which forms toroidal pores, can also dissolve the membrane through the carpet mechanism at highly critical concentrations (Oren and Shai 1998), suggesting that the carpet model is an extreme consequence of the toroidal pore mechanism (Brogden 2005).

C.3.2. Inhibition of intracellular targets

Although disruption of the cytoplasmic membrane integrity is the principal mechanism of AMP-induced cell death, many examples have been identified of AMPs that kill microbes without compromising the membrane, and of AMPs whose membrane-disruptive mechanisms are not sufficient to justify their antimicrobial activity. In these cases, such AMPs were revealed to target and inhibit intracellular components and enzymatic pathways important for cell viability (Ganz and Lehrer 1995, Yeaman and Yount 2003, Brogden 2005).

Peptidoglycan is a unique bacterial structure that confers physical support and protection. Therefore, AMPs targeting its biosynthetic machinery are regarded as highly effective killing agents. As mentioned before, lantibiotics were shown to inhibit cell wall synthesis as a consequence of using lipid II as membrane receptor, hijacking it from both peptidoglycan and WTA biosynthesis pathways (Islam *et al.*

2012). Recently, the defensin HNP-1 was also reported to act through this same mechanism (de Leeuw *et al.* 2010). Furthermore, lantibiotics like nisin and lacticin 3147 use this receptor as a platform for assembling transmembrane pores, leading to the leakage of intracellular content and membrane destabilization (Islam *et al.* 2012). Nisin and Pep5, another lantibiotic, were also shown to stimulate autolytic activity in *Staphylococcus simulans* (Bierbaum and Sahl 1987), which could result in uncontrolled cell wall lysis and death.

On the other hand, the toad-derived linear α-helical buforin II was found to cross the cytoplasmic membrane without compromising its integrity and accumulate in the cytoplasm, where it targets nucleic acids (Park *et al.* 1998, Park *et al.* 2000) possibly by binding to histone H2A (Cho *et al.* 2009).

Previous studies detected AMP molecules on the cytoplasmic side of the membrane after disassembly of transient toroidal pores, suggesting this as a mechanism to translocate AMPs across the membrane to further interact with intracellular targets (Uematsu and Matsuzaki 2000). This appears to be the case of buforin II, where a proline residue plays a critical role in membrane translocation through the formation of short-lived toroidal pores (Elmore 2012). Similarly, the cathelicidins indolicidin and PR-39 kill bacteria by accessing to their cytoplasm, without lysing the membrane, and interfering with both protein and DNA synthesis (Boman *et al.* 1993, Subbalakshmi and Sitaram 1998). Moreover, these two peptides were found to induce bacterial filamentation (Shi *et al.* 1996, Subbalakshmi and Sitaram 1998), which could be a consequence of DNA replication stalling.

Pyrrhocoricin and apidaecin, two small proline-rich insect AMPs that target mostly Gram-negative bacteria, bind to the bacterial chaperone proteins DnaK and GroEL, inhibiting their protein folding assistance activity (Otvos *et al.* 2000, Kragol *et al.* 2001). It is suspected that apidaecin might be translocated into the cytoplasm by a process similar to pediocin-like bacteriocins, i.e. through binding to an IM permease/transport component (Castle *et al.* 1999).

The Gram-negative class I microcins are another example of AMPs that cross the cytoplasmic membrane harmlessly to interfere with nucleic acid synthesis (Duquesne and Destoumieux-Garzón 2007).

C.4. Bacterial mechanisms of resistance

Millions of years of co-existence enabled bacterial pathogens to evolve strategies to resist against host defense mechanisms and effectors like AMPs (Peschel and Sahl 2006). In general, these strategies prevent AMPs to accomplish either of the following steps: (i) reach or (ii) attach to the bacterial surface, (iii) irreversibly permeabilize or (iv) translocate the cytoplasmic membrane; or (v) inhibit vital intracellular processes (Yeaman and Yount 2003, Brogden 2005).

As a first line of protection, bacteria may secrete proteins that inactivate AMPs by proteolysis. Linear α-helical peptides, such as LL-37, are particularly susceptible to the activity of proteases from both Gram-negative and Gram-positive species because of their exposed backbone (Resnick *et al.* 1991, Guina *et al.* 2000, Schmidtchen *et al.* 2002, Belas *et al.* 2004, Nyberg *et al.* 2004, Sieprawska-Lupa *et al.* 2004, Kooi and Sokol 2009). In contrast, AMPs with a more packed conformation or containing structurally stabilizing features like intramolecular disulfide bridges (e.g. defensins and protegrins), thioether bonds (lantibiotics) or abundant proline residues are considerably less prone to proteolysis (Peschel and Sahl 2006). Moreover, secreted bacterial proteins may also neutralize AMPs by binding and entrapping them before they reach the bacterial surface, as exemplified by staphylokinase and streptococcal M1 and SIC proteins (Frick *et al.* 2003, Jin *et al.* 2004, Lauth *et al.* 2009). Alternatively, some bacteria are able to assemble a capsule around their surface, which physically blocks AMPs from interacting with the membrane. Indeed, unencapsulated strains have showed higher sensitivity to AMPs than their wild-type capsulated congeners (Campos *et al.* 2004, Llobet *et al.* 2008).

Despite these tactics, the majority of the bacterial defense mechanisms against AMPs involve changes in the architecture or composition of the bacterial cell envelope to render it less vulnerable to AMP attachment or penetration.

C.4.1. Modification of cell envelope components

As previously mentioned, a unique bacterial characteristic explored by AMPs to exert their activity is the net negative electrostatic charge of the cell envelope,

which promotes AMP attraction and association due to their cationic nature. Therefore, a major defensive strategy employed by both Gram-negative and Gram-positive bacteria is to biochemically adjust the surface charge to reduce its electronegativity and concomitantly weaken AMP affinity towards it (Peschel 2002). This is generally accomplished by the addition of molecules containing free protonated amino groups to the structure of phosphate-rich surface components, whereby the positive charge of the amino group would cancel the negative charge of a nearby phosphate group.

In Gram-negative bacteria, this modification procedure is performed on the lipid A moiety of LPS, an OM-linked glycopolymer and one of the main AMP attractors to the bacterial surface. In this case, aminated compounds such as aminoarabinose (Stinavage *et al.* 1989, Guo *et al.* 1997) and ethanolamine (Zhou *et al.* 2001, Tran *et al.* 2006) are appended to the lipid A phosphate groups to mask their negative charge. The importance of these alterations towards bacterial AMP resistance is attested by the reduced *in vivo* virulence of mutant strains lacking either of these LPS modification mechanisms (Gunn *et al.* 2000, Cullen *et al.* 2011). Alternatively, lipid A acyl chains can be esterified with glycine (Hankins *et al.* 2012) or with additional fatty acids, which appears to promote AMP resistance by producing a membrane less fluid and permissive for AMP insertion and disruption (Guo *et al.* 1997, Brogden 2005). Again, failure to perform this process yields strains with increased AMP susceptibility (Guo *et al.* 1998, Robey *et al.* 2001).

TAs are the major phosphate-rich cell surface components in Gram-positive bacteria, and thus the primary target of surface charge modulation mechanisms. D-alanine esters are highly common TA substituents and the introduction of these aminated groups contribute significantly to the masking of the Gram-positive surface electronegativity and for increased resistance to different AMPs (Fig. 18) (Peschel *et al.* 1999, Abachin *et al.* 2002, Collins *et al.* 2002, Kristian *et al.* 2005, Kovacs *et al.* 2006, Abi Khattar *et al.* 2009, McBride and Sonenshein 2011). Interestingly, a recent study in *Streptococcus agalactiae* proposed that D-alanylation of TAs confers resistance to AMPs preferentially by hindering their penetration through the cell wall than by influencing their initial binding to the bacterial surface (Saar-Dover *et al.* 2012).

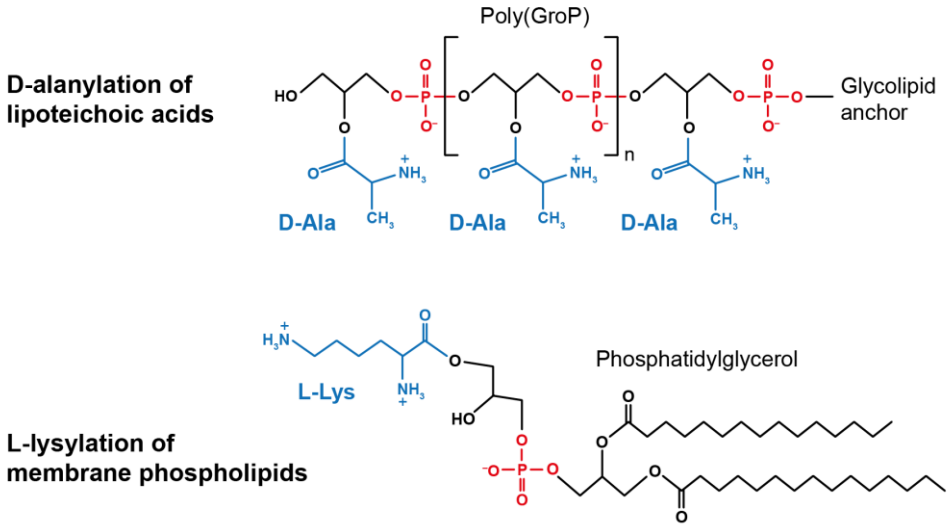

Fig. 18. Mechanisms of charge modulation of *Lm* cell envelope components. The anionic character of *Lm* cell envelope components such as TAs or some membrane phospholipids (negatively charged phosphate groups in red) can be masked with the addition of positively charged molecules (blue). These modifications typically involve esterification with certain amino acids, where protonated amino groups contain a positive charge. In *Lm*, LTAs undergo D-alanylation through the action of the Dlt pathway (Neuhaus and Baddiley 2003), while the head group of phosphatidylglycerol is substituted with L-lysine by the action of MprF, leading to a charge reversal (-1 to +1).

Alternatively, positively charged amino acid esters can also be linked to the head groups of outer leaflet membrane phospholipids. In *Lm*, this enzymatic reaction is catalyzed by a multiple peptide resistance factor (MprF) protein that transfers L-lysine from cytoplasmic tRNA precursors to the terminal glycerol of PG, forming lysyl-PG, which unlike PG has a net positive charge (+1) due to the two protonated amino groups of L-lysine (Fig. 18). Similarly to TA D-alanylation, absence of MprF-mediated lysyl-PG generation compromises bacterial survival when challenged with AMPs, as observed with *S. aureus* and *Lm* (Peschel *et al.* 2001, Kristian *et al.* 2003, Thedieck *et al.* 2006, Andra *et al.* 2011).

O-acetylation is a widespread post-assembly peptidoglycan modification mechanism (Vollmer 2008). It is catalyzed by an integral membrane *O*-acetyltransferase that captures acetyl-containing substrates from the cytoplasm and transfers the acetyl group to the C6-linked hydroxyl group of Mur*N*Ac residues in the assembled peptidoglycan strands (Clarke *et al.* 2002). First discovered in *S. aureus* (Bera *et al.* 2005), orthologue genes coding for such an enzyme were recently identified also in *Lm* (Aubry *et al.* 2011) and *H. pylori* (Wang *et al.* 2012). This modification was demonstrated to favor bacterial resistance against the

muramidase activity of lysozyme (Dupont and Clarke 1991, Bera *et al.* 2006, Veiga *et al.* 2007, Aubry *et al.* 2011, Guariglia-Oropeza and Helmann 2011, Wang *et al.* 2012). Interestingly, *Lm* mutant strains lacking this enzyme, thus devoid of *O*-acetylated peptidoglycan, showed increased vulnerability to the lantibiotic gallidermin (Aubry *et al.* 2011).

CHAPTER II

PROJECT PRESENTATION

The main goal of our research group is to expand the knowledge on the molecular mechanisms employed by *Lm* to interact with a host organism and promote pathogenesis. To achieve this, it is essential to have a global perspective of how this bacterial pathogen behaves in the context of infection, of how it reacts and adapts to the multiple biological cues – favorable and harmful – within a susceptible host. In this sense, a couple of studies performed array-based analyses of the transcriptional response of *Lm* during infection of cell lines *in vitro*, in order to identify genes that were important for invasion, survival and proliferation within host cells (Chatterjee *et al.* 2006, Joseph *et al.* 2006). However, unlike what had been done with other pathogenic bacteria (La *et al.* 2008), no information was available regarding the transcriptional profiling of *Lm* in a natural infection context, i.e. inside a living host organism.

To fill this void, we performed the first *in vivo* transcriptome analysis of *Lm*, where whole-genome expression changes in bacteria infecting the mouse spleen were compared against those of bacteria growing *in vitro* (Camejo *et al.* 2009) (see publication in Chapter VI). The resulting data showed that *Lm* modifies the expression levels of about 20% of its genome throughout infection, mostly by gene upregulation. All the major virulence-associated genes and regulators were found to be highly transcribed, as expected, but several other genes with uncharacterized functions or with no previously known connection with *Lm* pathogenesis were also activated (Camejo *et al.* 2009). Among these uncharacterized genes, those lacking orthologues in non-virulent *Listeria* species (such as *L. innocua*), were of particular interest for their potential involvement in *Lm* infection.

Included in this group were four contiguous genes – *lmo1081* to *lmo1084* – that displayed significant overexpression *in vivo*. Moreover, the encoded proteins were annotated as homologues of the products of the *rmlABCD* gene cluster, which catalyze the metabolic pathway responsible for the biosynthesis of L-rhamnose (Giraud and Naismith 2000). Interestingly, this monosaccharide is produced in bacteria but not in animals (Tonetti *et al.* 1998). However, even more striking is its presence at the cell surface of several pathogenic bacteria, where it can be found associated with important virulence structures such as the LPS O-antigen, rhamnolipids or the mycobacterial arabinogalactan (Ma *et al.* 2001,

Samuel and Reeves 2003, Zulianello *et al.* 2006). These observations led us to consider that the *Lm* equivalent of this *rmlABCD* cluster might also be involved in virulence-promoting processes.

Therefore, the aims of this work were to **determine the purpose of the *rmlABCD* cluster homologue in *Lm* biology** and **assess its potential contribution to *Lm* virulence**. For this, relevant mutant strains were generated and analyzed *in vitro* with the intention of addressing the main biochemical role of the cluster-encoded proteins in *Lm*. The elucidation of this role led to the investigation of further related mechanisms with key importance in aspects like *Lm* resistance against microbicidal molecules and surface protein anchoring. To evaluate the involvement of the cluster in *Lm* pathogenesis, these mutant strains were also tested *in vivo*, using the mouse model of infection.

CHAPTER III

RESULTS

The results produced by this work are presented in two parts:

- **PART I – L-Rhamnosylation of *Listeria monocytogenes* wall teichoic acids promotes resistance to antimicrobial peptides by delaying interaction with the membrane**

  Here, we describe the role of the *Lm rml* gene cluster, showing its requirement for L-rhamnosylation of *Lm* WTAs, and present functional and mechanistic evidences linking this event with bacterial resistance to AMPs. Ultimately, we confirm the important contribution of this particular WTA glycosylation mechanism to *Lm* pathogenesis.

  These findings were published on **PLoS Pathogens** (22 May 2015), and the published version is appended in Chapter VI.

- **PART II – L-Rhamnosylation of *Listeria monocytogenes* wall teichoic acids is required for efficient surface anchoring of GW proteins**

  In this part, we include unpublished data from ongoing work that highlight other important processes in *Lm* depending on the WTA L-rhamnosylation status, such as bacterial autolysis and invasion of host cells. We show that contribution to these events is supported by a newly identified role for L-rhamnosylated WTAs in the anchoring of a particular family of *Lm* surface proteins sharing similar cell surface-binding domains.

PART I

L-Rhamnosylation of *Listeria monocytogenes* wall teichoic acids promotes resistance to antimicrobial peptides by delaying interaction with the membrane

L-Rhamnosylation of *Listeria monocytogenes* Wall Teichoic Acids Promotes Resistance to Antimicrobial Peptides by Delaying Interaction with the Membrane

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I.1. Abstract

Listeria monocytogenes is an opportunistic Gram-positive bacterial pathogen responsible for listeriosis, a human foodborne disease. Its cell wall is densely decorated with wall teichoic acids (WTAs), a class of anionic glycopolymers that play key roles in bacterial physiology, including protection against the activity of antimicrobial peptides (AMPs). In other Gram-positive pathogens, WTA modification by amine-containing groups such as d-alanine was largely correlated with resistance to AMPs. However, in *L. monocytogenes* where WTA modification is achieved solely *via* glycosylation, WTA-associated mechanisms of AMP resistance were unknown. Here, we show that the L-rhamnosylation of *L. monocytogenes* WTAs relies not only on the *rmlACBD* locus, which encodes the biosynthetic pathway for L-rhamnose, but also on *rmlT* encoding a putative rhamnosyltransferase. We demonstrate that this WTA tailoring mechanism promotes resistance to AMPs, unveiling a novel link between WTA glycosylation and bacterial resistance to host defense peptides. Using *in vitro* binding assays, fluorescence-based techniques and electron microscopy, we show that the presence of L-rhamnosylated WTAs at the surface of *L. monocytogenes* delays the crossing of the cell wall by AMPs and postpones their contact with the listerial membrane. We propose that WTA L-rhamnosylation promotes *L. monocytogenes* survival by decreasing the cell wall permeability to AMPs, thus hindering their access and detrimental interaction with the plasma membrane. Strikingly, we reveal a key contribution of WTA L-rhamnosylation for *L. monocytogenes* virulence in a mouse model of infection.

I.2. Author Summary

Listeria monocytogenes is a foodborne bacterial pathogen that preferentially infects immunocompromised hosts, eliciting a severe and often lethal disease. In humans, clinical manifestations range from asymptomatic intestinal carriage and gastroenteritis to harsher systemic states of the disease such as sepsis, meningitis or encephalitis, and fetal infections. The surface of *L. monocytogenes* is decorated with wall teichoic acids (WTAs), a class of carbohydrate-based polymers that contributes to cell surface-related events with implications in physiological processes, such as bacterial division or resistance to antimicrobial peptides (AMPs). The addition of other molecules to the backbone of WTAs modulates their chemical properties and consequently their functionality. In this context, we studied the role of WTA tailoring mechanisms in *L. monocytogenes*, whose WTAs are strictly decorated with monosaccharides. For the first time, we link WTA glycosylation with AMP resistance by showing that the decoration of *L. monocytogenes* WTAs with L-rhamnose confers resistance to host defense peptides. We suggest that this resistance is based on changes in the permeability of the cell wall that delay its crossing by AMPs and therefore promote the protection of the bacterial membrane integrity. Importantly, we also demonstrate the significance of this WTA modification in *L. monocytogenes* virulence.

I.3. Introduction

Listeria monocytogenes (*Lm*) is a ubiquitous Gram-positive bacterium and the causative agent of listeriosis, a human foodborne disease with high incidence and morbidity in immunocompromised hosts and other risk groups, such as pregnant women, neonates and the elderly. Clinical manifestations range from febrile gastroenteritis to septicemia, meningitis and encephalitis, as well as fetal infections that can result in abortion or postnatal health complications (Swaminathan and Gerner-Smidt 2007b). The most invasive and severe forms of the disease are a consequence of the ability of this pathogen to overcome important physiological barriers (intestinal epithelium, blood-brain barrier and placenta) by triggering its internalization and promoting its intracellular survival into phagocytic and non-phagocytic cells. Once inside a host cell, a tightly coordinated life cycle, whose progression is mediated by several specialized bacterial factors, enables *Lm* to proliferate and spread to neighboring cells and tissues (Cossart and Toledo-Arana 2008, Camejo *et al.* 2011).

The *Lm* cell wall is composed of a thick peptidoglycan multilayer that serves as a scaffold for the anchoring of proteins, among which are several virulence factors (Carvalho *et al.* 2014) (see publication in Chapter VI), and of glycopolymers such as teichoic acids, which account for up to 70% of the protein-free cell wall mass (Fiedler *et al.* 1984, Fiedler 1988). These anionic polymers are divided into membrane-anchored teichoic acids (lipoteichoic acids, LTAs) and peptidoglycan-attached teichoic acids (wall teichoic acids, WTAs). In *Listeria*, WTAs are mainly composed of repeated ribitol-phosphate subunits, whose hydroxyl groups can be substituted with a diversity of monosaccharides (Fiedler *et al.* 1984). While the polymer structure and the chemical identity of the substituent groups of LTAs are rather conserved across listeriae (Uchikawa *et al.* 1986b, Ruhland and Fiedler 1987), they display a high variability in WTAs, even within the same species (Weidenmaier and Peschel 2008). Specific WTA substitution patterns are characteristic of particular *Lm* serotypes: *N*-acetylglucosamine is common to serogroups 1/2 and 3, and to serotype 4b, but serogroup 1/2 also contains L-rhamnose, whereas serotype 4b displays D-glucose and D-galactose (Uchikawa *et al.* 1986a). The broad structural and chemical similarity of LTAs and

WTAs results in a considerable degree of functional redundancy, which has complicated the characterization of these macromolecules and the assignment of specific biological roles. However, studies on Gram-positive bacteria have revealed their contribution to important physiological functions (e.g. cell envelope cationic homeostasis (Marquis *et al.* 1976), regulation of autolysin activity (Peschel *et al.* 2000), assembly of cell elongation and division machineries (Schirner *et al.* 2009), defense against antimicrobial peptides (Peschel *et al.* 1999)) and to virulence-promoting processes, such as adhesion and colonization of host tissues (Weidenmaier *et al.* 2004, Weidenmaier *et al.* 2005).

Antimicrobial peptides (AMPs) are a large family of small peptides (<10 kDa) produced by all forms of living organisms (Cederlund *et al.* 2011), which constitute a major player of the innate immune response against microbial pathogens. Despite their structural diversity, the majority of AMPs share both cationic and amphipathic properties that favor respectively their interaction with the negatively charged prokaryotic surface and insertion into the plasma membrane (Peters *et al.* 2010, Cederlund *et al.* 2011). Subsequent pore formation or other AMP-mediated membrane-disrupting mechanisms induce bacterial death through direct cell lysis or deleterious interaction with intracellular targets (Brogden 2005). Bacteria have evolved multiple strategies to avert killing by AMPs (Peschel and Sahl 2006, Koprivnjak and Peschel 2011). One strategy consists in the modification of their cell surface charge, a process achieved mainly by masking anionic glycopolymers with positively charged groups, thus decreasing their affinity to AMPs. In Gram-positive pathogens, D-alanylation of teichoic acids is a well-characterized mechanism and was demonstrated to be important for bacterial resistance to host-secreted AMPs (Koprivnjak *et al.* 2002, Neuhaus and Baddiley 2003). In contrast, the contribution of WTA glycosylation mechanisms in AMP resistance has not yet been investigated.

We have previously reported genome-wide transcriptional changes occurring in *Lm* strain EGD-e during mouse infection (Camejo *et al.* 2009). Our analysis revealed an elevated *in vivo* expression of the *lmo1081-1084* genes, here renamed as *rmlACBD* because of the high homology of the corresponding proteins with enzymes of the L-rhamnose biosynthesis pathway. In this work, we show that the decoration of *Lm* WTAs with L-rhamnose requires the expression of not only

the *rmlACBD* locus but also of *rmlT*, an upstream-flanking gene encoding a putative rhamnosyltransferase. We also demonstrate that *Lm* becomes more susceptible to AMPs in the absence of WTA L-rhamnosylation and predict that this effect is due to an increase of the *Lm* cell wall permeability to these bactericides, which results in a faster disruption of the plasma membrane integrity with lethal consequences for the bacterial cell. Importantly, we present evidence that this WTA tailoring process is required for full-scale *Lm* virulence in the mouse model of infection.

I.4. Results

I.4.1. The *rmlACBD* locus is required for the presence of L-rhamnose in *Lm* WTAs

To identify new *Lm* genes potentially critical for the infectious process, we previously performed the first *in vivo* transcriptional profiling of *Lm* EGD-e. Among the *Lm* genes displaying the largest increase in transcription throughout infection, we identified a set of previously uncharacterized genes that are included in a pentacistronic operon (*lmo1080* to *lmo1084*) (Toledo-Arana *et al.* 2009). This operon is found in *L. monocytogenes* strains belonging to serogroups 1/2, 3 and 7, and is absent from serogroup 4 strains (Doumith *et al.* 2004) (Fig. 19). Interestingly, aside from *Listeria seeligeri* 1/2b strains, this locus is not found in any other *Listeria* spp., such as the nonpathogenic *Listeria innocua* or the ruminant pathogen *Listeria ivanovii*, which pinpoints it as a genetic feature of a particular subset of pathogenic *Listeria* strains and suggests that its expression may be important to *Listeria* pathogenesis in humans.

The four proteins encoded by the *lmo1081-lmo1084* genes share a high amino acid sequence homology with the products of the *rmlABCD* gene cluster. These genes are widely distributed among Gram-negative (e.g. *Salmonella enterica* (Li and Reeves 2000), *Shigella flexneri* (Macpherson *et al.* 1994), *Vibrio cholerae* (Li *et al.* 2003), *Pseudomonas aeruginosa* (Aguirre-Ramírez *et al.* 2012)) and Gram-positive species (e.g. *Mycobacterium tuberculosis* (Li *et al.* 2006), *Streptococcus mutans* (Tsukioka *et al.* 1997), *Geobacillus tepidamans* (Zayni *et al.* 2007), *Lactobacillus rhamnosus* (Péant *et al.* 2005)) (Fig. 19), the majority of which being known pathogens or potentially pathogenic. Despite the inter-species variability observed in the genetic organization of the *rml* genes, the respective proteins exhibit a remarkable degree of conservation (Table S1). In light of this, we renamed the *lmo1081-lmo1084* genes to *rmlACBD*, respectively (Fig. 19).

The RmlABCD proteins catalyze the conversion of glucose-1-phosphate to a thymidine-diphosphate (dTDP)-linked form of L-rhamnose (Giraud and Naismith 2000) (Fig. S1A), which is a component of the WTAs from most *Listeria* strains possessing the *rml* genes (Fiedler 1988). To address the role of *rmlACBD* in *Lm* WTA glycosylation with L-rhamnose, we constructed an *Lm* EGD-e derivative

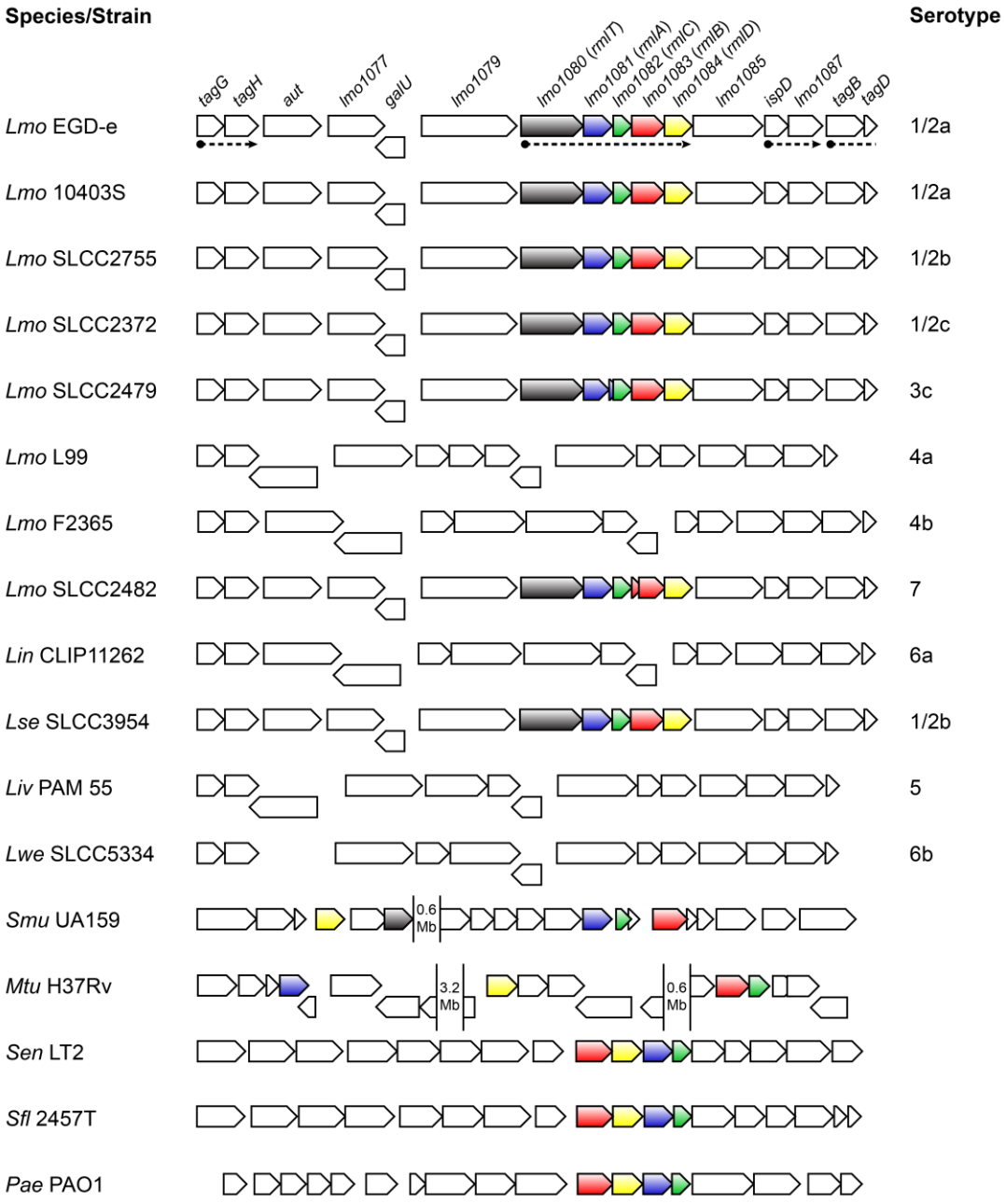

Fig. 19. **Genes encoding the L-rhamnose biosynthesis pathway are distributed in listeriae and other bacterial species.** Comparison of the genomic organization of the L-rhamnose pathway genes in the genus *Listeria* and other bacteria. The corresponding species and strains are indicated on the left (*Lmo*, *Listeria monocytogenes*; *Lin*, *Listeria innocua*; *Lse*, *Listeria seeligeri*; *Liv*, *Listeria ivanovii*; *Lwe*, *Listeria welshimeri*; *Smu*, *Streptococcus mutans*; *Mtu*, *Mycobacterium tuberculosis*; *Sen*, *Salmonella enterica* serovar Typhimurium; *Sfl*, *Shigella flexneri*; *Pae*, *Pseudomonas aeruginosa*) and listerial serotypes are indicated on the right. Genes are represented by boxed arrows and their names are provided for strain EGD-e. Operons are underlined by dashed arrows and homologs of the *rml* genes are shown with identical colors. Numbered gaps indicate the genetic distance (Mb, mega base pairs) between *rml* genes located far apart in the chromosome. Bacterial genomic sequences were obtained from NCBI database and chromosomal alignments assembled using Microbial Genomic context Viewer and Adobe Illustrator.

mutant strain lacking the *rmlACBD* locus (Δ*rmlACBD*) (Fig. S2A) and investigated if the absence of these genes could affect the WTA L-rhamnosylation status. We

prepared WTA hydrolysates from exponential phase cultures of wild type (EGD-e), Δ*rmlACBD* and a complemented Δ*rmlACBD* strain expressing *rmlACBD* from its native promoter within an integrative plasmid (Δ*rmlACBD*+*rmlACBD*). Samples were resolved by native PAGE and the gel stained with Alcian blue to visualize WTA polymer species. A mutant strain unable to synthesize WTAs (Δ*tagO1*Δ*tagO2*) (Eugster and Loessner 2012) was used to confirm that the detected signal corresponds to WTAs. Compared to the wild type sample, the Δ*rmlACBD* WTAs displayed a shift in migration, which was reverted to a wild type-like profile in WTAs from the Δ*rmlACBD*+*rmlACBD* sample (Fig. 20A), indicating that the native WTA composition requires the presence of the *rmlACBD* genes. To confirm this, we investigated the WTA carbohydrate composition from these strains. WTA polymers were isolated from cell walls purified from bacteria in exponential growth phase, hydrolyzed and analyzed by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) to detect monosaccharide species. WTA extracts obtained from Δ*rmlACBD* bacteria completely lacked L-rhamnose, in contrast to those isolated from the parental wild type strain (Fig. 20B). The role of *rmlACBD* in *Lm* WTA L-rhamnosylation was definitely confirmed by the analysis of WTAs from Δ*rmlACBD*+*rmlACBD* bacteria, in which L-rhamnose was detected at levels similar to those observed in the wild type sample (Fig. 20B). Similar observations were made with purified cell wall samples that contain WTAs still attached to the peptidoglycan matrix (Fig. S3A). The absence of muramic acid, one of the peptidoglycan building blocks, from WTA extracts (Fig. 20B) indicates that L-rhamnose is specifically associated with WTAs and is not a putative peptidoglycan contaminant. This is corroborated by the absence of L-rhamnose in purified peptidoglycan samples (Fig. 20C).

WTAs have been identified as important regulators of peptidoglycan cross-linking and maturation (Atilano *et al.* 2010). To investigate if L-rhamnose decoration of WTAs has any involvement in the maturation of the *Lm* peptidoglycan, we performed HPLC analysis of the muropeptide composition of mutanolysin-digested peptidoglycan samples from wild type, Δ*rmlACBD* and Δ*rmlACBD*+*rmlACBD* bacteria. No differences in the nature and relative amount of muropeptide species were observed between strains (Fig. S3B), ruling out a role

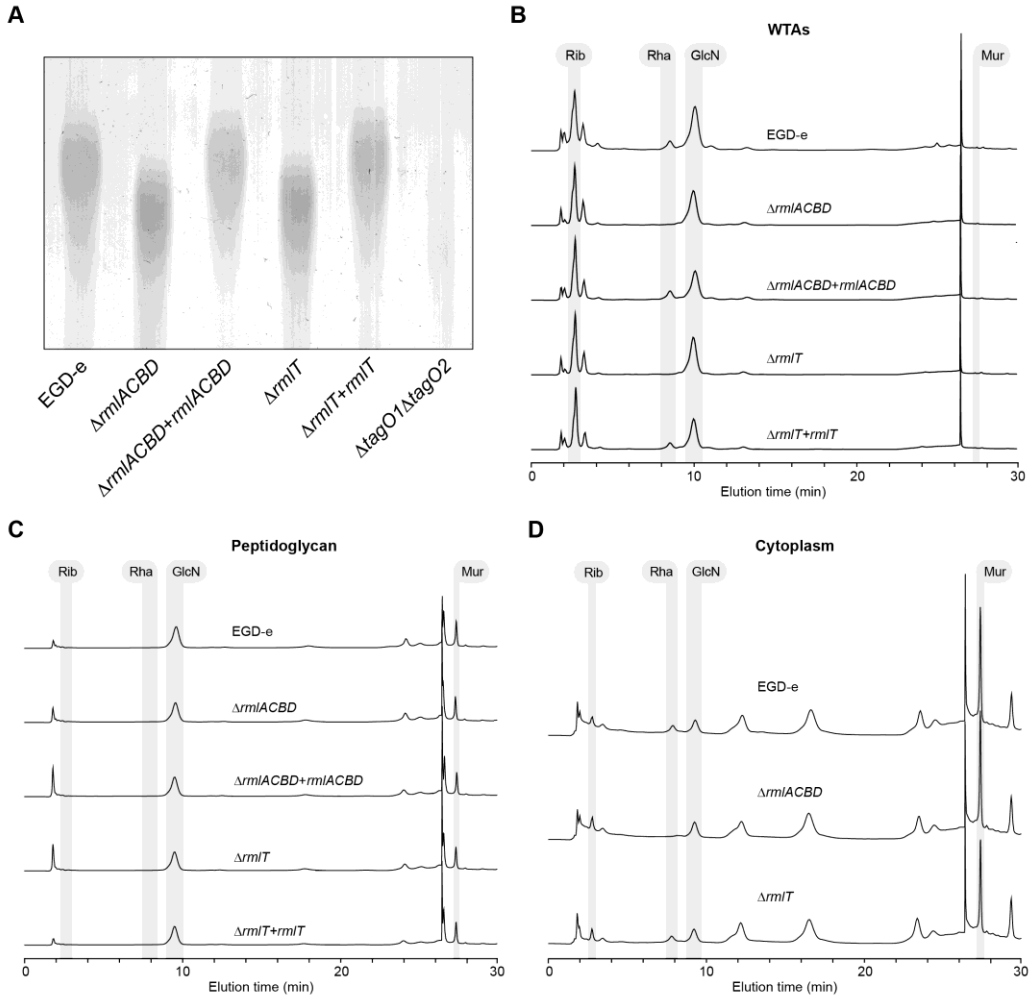

Fig. 20. **A functional *rml* operon is required for glycosylation of *Lm* WTAs with L-rhamnose.** (**A**) Alcian blue-stained 20% polyacrylamide gel containing WTA extracts from logarithmic-phase cultures of different *Lm* strains. (**B–D**) HPAEC-PAD analyses of the sugar composition of the (B) WTA, (C) peptidoglycan and (D) cytoplasmic fractions isolated from the indicated *Lm* strains. Samples were hydrolyzed in 3 M HCl (2 h, 95 ºC), diluted with water and lyophilized before injection into the HPLC equipment. Standards for ribitol (Rib), L-rhamnose (Rha), glucosamine (GlcN), and muramic acid (Mur) were eluted under identical conditions to allow peak identification.

for WTA L-rhamnosylation in the consolidation of the peptidoglycan architecture. Overall, these results confirm that a functional *rmlACBD* locus is required for the association of L-rhamnose with *Lm* WTAs, likely by providing the molecular machinery responsible for the synthesis of L-rhamnose.

I.4.2. RmlT is required for the incorporation of L-rhamnose into *Lm* WTAs

The *rml* operon in *Lm* includes a fifth gene, *lmo1080*, located upstream of *rmlA* (Fig. 19), which codes for a protein similar to the *B. subtilis* minor teichoic

acid biosynthesis protein GgaB, shown to possess sugar transferase activity (Freymond *et al.* 2006). Conserved domain analysis of the translated Lmo1080 amino acid sequence revealed that its N-terminal region is highly similar (e-value 10^{-22}) to a GT-A family glycosyltransferase domain (Fig. S1B). In GT-A enzymes, this domain forms a pocket that accommodates the nucleotide donor substrate for the glycosyl transfer reaction, and contains a signature DxD motif necessary to coordinate a catalytic divalent cation (Breton *et al.* 2006). This motif is also found within the predicted glycosyltransferase domain sequence of Lmo1080 as a DHD tripeptide (Fig. S1B). For these reasons, we investigated whether Lmo1080, which we renamed here RmlT (for L-rhamnose transferase), was involved in the L-rhamnosylation of *Lm* WTAs. We constructed an *Lm* EGD-e mutant strain lacking *rmlT* (Fig. S2A) and analyzed the structure and sugar composition of its WTAs as described above. WTAs isolated from Δ*rmlT* bacteria displayed a faster migration in gel (Fig. 20A) and did not contain any trace of L-rhamnose (Fig. 20B), fully recapitulating the Δ*rmlACBD* phenotype. Reintroduction of a wild type copy of *rmlT* into the mutant strain (Δ*rmlT*+*rmlT*) resulted in a phenotype that resembles that of the wild type strain, with regards to WTA gel migration profile (Fig. 20A) and presence of L-rhamnose in the WTA fraction (Fig. 20B).

To discard the possibility that the deletion of *rmlT* exerted a negative polar effect on the downstream expression of *rmlACBD*, potentially disrupting the synthesis of L-rhamnose used for WTA glycosylation, we compared the transcription of the *rmlACBD* genes in the wild type and Δ*rmlT Lm* strains by quantitative real-time PCR. Transcript levels were unchanged in the Δ*rmlT* background as compared to the wild type strain (Fig. S2B), indicating that the deletion of *rmlT* did not interfere with the transcription of *rmlACBD*. To definitely confirm that *Lm* Δ*rmlT* still holds the capacity to synthesize L-rhamnose, being only incapable to incorporate it in nascent WTA polymers, we evaluated the presence of L-rhamnose in the cytoplasmic compartment of this strain. The intracellular content of early exponential-phase bacteria from the wild type, Δ*rmlACBD* and Δ*rmlT* strains was extracted, hydrolyzed and analyzed by HPAEC-PAD to compare the sugar composition of cytoplasmic extracts. As shown in Fig. 20D, a peak corresponding to L-rhamnose was detected in the cytoplasmic samples from the wild type and Δ*rmlT* strains, but not from the Δ*rmlACBD* strain, clearly

demonstrating that, as opposed to Δ*rmlACBD* bacteria, Δ*rmlT* bacteria retain a functional L-rhamnose biosynthesis pathway. These results indicate that the depletion of L-rhamnose observed in Δ*rmlT* WTAs is a consequence of the absence of the WTA L-rhamnosyltransferase activity performed by RmlT. Therefore, we propose RmlT as the glycosyltransferase in charge of decorating *Lm* WTAs with L-rhamnose.

I.4.3. WTA L-rhamnosylation promotes *Lm* resistance to AMPs

WTAs were previously associated with bacterial resistance against salt stress (Chassaing and Auvray 2007) and host defense effectors, such as lysozyme (Bera *et al.* 2007, Atilano *et al.* 2010). We thus investigated the potential involvement of WTA L-rhamnosylation in these processes by assessing the growth of the Δ*rmlACBD* and Δ*rmlT* strains in the presence of high concentrations of either NaCl or lysozyme. As shown in Fig. 21A, no significant difference was observed between the growth of the wild type and the two mutant strains in BHI broth containing 5% NaCl. Similarly, no difference was detected between the growth behavior of these strains after the addition of different concentrations of lysozyme (50 µg/ml and 1 mg/ml) to bacterial cultures in the exponential phase (Fig. 21B). As expected, we observed an immediate and significant decrease in the survival of the lysozyme-hypersensitive Δ*pgdA* mutant (Boneca *et al.* 2007) (Fig. 21B). These data demonstrate that *Lm* does not require L-rhamnosylated WTAs to grow under conditions of high osmolarity nor to resist the cell wall-degrading activity of lysozyme.

WTAs were also found to be involved in bacterial resistance to host-secreted defense peptides (Peschel *et al.* 1999, Kovacs *et al.* 2006). To investigate the role of WTA L-rhamnosylation in *Lm* resistance to AMPs, we evaluated the *in vitro* survival of wild type, Δ*rmlACBD* and Δ*rmlT Lm*, as well as of the respective complemented strains, in the presence of biologically active synthetic forms of AMPs produced by distinct organisms: gallidermin, a bacteriocin from the Gram-positive bacterium *Staphylococcus gallinarum* (Kellner *et al.* 1988); CRAMP, a mouse cathelicidin (Gallo *et al.* 1997), or its human homolog LL-37 (Vandamme *et al.* 2012). After two hours of co-incubation with different AMP concentrations,

surviving bacteria were enumerated by plating in solid media. The overall survival levels of *Lm* varied with each AMP, evidencing their distinct antimicrobial effectiveness (Fig. S4). However, when compared to the wild type strain, the Δ*rmlACBD* and Δ*rmlT* mutants displayed a consistent decrease in their survival levels in the presence of any of the three AMPs (Fig. 21C), in a dose-dependent manner (Fig. S4). Restoring WTA L-rhamnosylation through genetic complementation of the mutant strains resulted in an increase of the survival rate to wild type levels. This result demonstrated the important contribution of L-rhamnosylated WTAs towards *Lm* resistance against AMPs, pointing to a role for WTA glycosylation in bacterial immune evasion mechanisms.

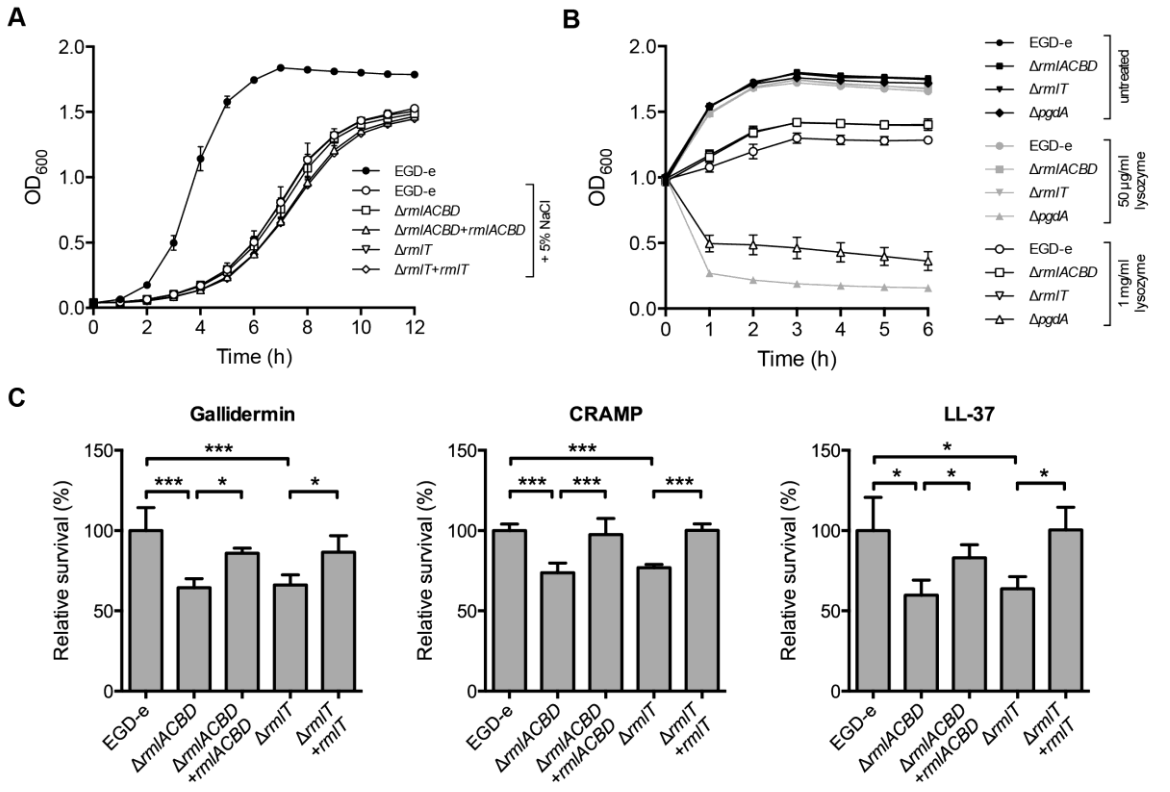

Fig. 21. **WTA L-rhamnosylation promotes *Lm* resistance against AMPs.** (**A**) Growth of *Lm* strains in BHI broth supplemented with 5% NaCl. A growth curve of wild type EGD-e in the absence of 5% NaCl was included as a control for optimal growth. (**B**) Growth of mid-exponential-phase *Lm* strains untreated (black symbols) or challenged with 50 µg/ml (gray symbols) or 1 mg/ml (white symbols) of lysozyme. Optical density of the shaking cultures was monitored spectrophotometrically at 600 nm. (**C**) Quantification of viable bacteria after treatment of mid-exponential-phase *Lm* strains (2 h, 37 ºC) with gallidermin (1 µg/ml), CRAMP or LL-37 (5 µg/ml). Averaged replicate values from AMP-treated samples were normalized to untreated control samples and the transformed data expressed as the percentage of surviving bacteria relative to wild type *Lm* (set at 100). Data represent mean±SD of three independent experiments. *, $p \leq 0.05$; ***, $p \leq 0.001$.

I.4.4. WTA L-rhamnosylation interferes with *Lm* cell wall crossing by AMPs

The increased AMP susceptibility of *Lm* strains defective in WTA L-rhamnosylation suggests that this process is required to hinder the bactericidal activity of AMPs. Since AMPs generally induce bacterial death by disrupting the integrity of the plasma membrane, we hypothesized that the higher susceptibility of the Δ*rmlACBD* and Δ*rmlT* mutant strains resulted from an increased AMP-mediated destabilization of the *Lm* membrane. In this context, two scenarios were envisioned: i) AMPs could be binding with higher affinity to the L-rhamnose-deficient *Lm* cell wall, or ii) they could be crossing it at a faster pace, thus reaching the membrane more quickly than in wild type *Lm*. To explore these possibilities, we first investigated the binding affinity of the mouse cathelicidin CRAMP towards *Lm* cell walls depleted of L-rhamnose. For this, we incubated the different *Lm* strains with CRAMP for a short period and analyzed by flow cytometry the amount of *Lm*-bound peptide exposed at the cell surface and accessible for antibody recognition. We detected fluorescence associated with surface-exposed CRAMP in all strains (Fig. 22A). However, the mean fluorescence intensity (MFI) values were significantly reduced in both Δ*rmlACBD* and Δ*rmlT* mutants, in comparison to wild type *Lm* and the complemented strains (Figs. 22A and 22B). This suggests that CRAMP was less accessible to immunolabeling at the cell surface of *Lm* lacking L-rhamnosylated WTAs.

The affinity of AMPs towards the bacterial surface is driven by electrostatic forces between positively charged peptides and the anionic cell envelope (Koprivnjak *et al.* 2002). To determine if variations of the *Lm* surface charge contributed to the reduced amount of CRAMP exposed at the surface of Δ*rmlACBD* and Δ*rmlT* bacteria, we compared the surface charge of *Lm* with or without L-rhamnosylated WTAs. For this, we analyzed the binding of cytochrome c, a small protein with positive charge at physiological conditions (isoelectric point ~10), to the wild type and mutant *Lm* strains. As positive control, we used a mutant strain that cannot modify its LTAs with D-alanine (Δ*dltA*) and, as a result, displays a higher surface electronegativity and a concomitant higher affinity for positively charged compounds (Peschel *et al.* 1999, Abachin *et al.* 2002). As expected, the level of cytochrome c binding was higher with the Δ*dltA* strain than with the

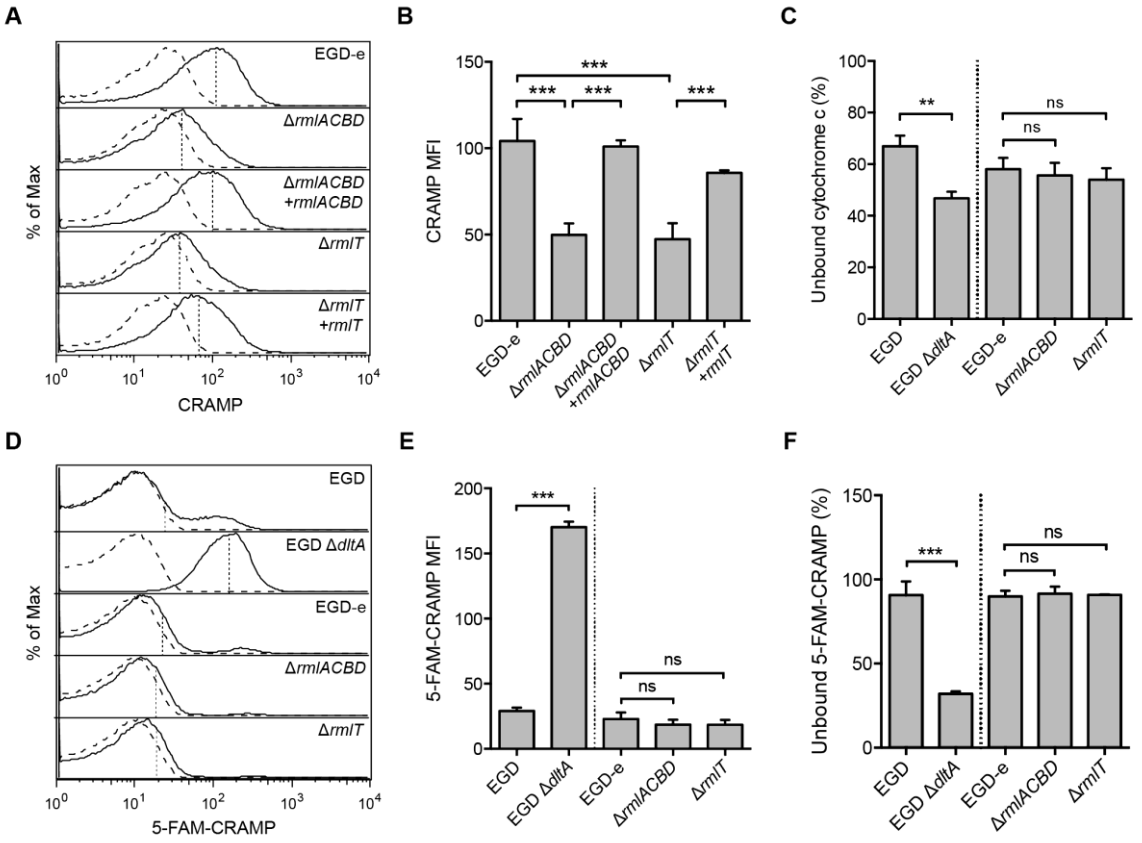

Fig. 22. **WTA L-rhamnosylation interferes with the *Lm* cell wall crossing by AMPs.** (**A and B**) Flow cytometry analysis of *Lm* surface-exposed CRAMP levels in mid-exponential-phase *Lm* strains, following incubation (5 min) in a 5-µg/ml solution of the peptide and immunolabeling with anti-CRAMP and Alexa Fluor 488-conjugated antibodies. (A) Representative experiment showing overlaid histograms of CRAMP-treated (solid line) and untreated (dashed line) samples, with mean fluorescence intensity (MFI) values from treated samples indicated by vertical dashed lines. (B) Mean±SD of the MFI values of CRAMP-treated samples from three independent experiments. (**C**) Cell surface charge analysis of *Lm* strains deficient for WTA L-rhamnosylation as determined by cytochrome c binding assays. Mid-exponential-phase bacteria were incubated with equine cytochrome c (0.5 mg/ml), centrifuged and the supernatant was recovered for spectrophotometric quantification of the unbound protein fraction. Values from *Lm*-containing samples are expressed as the percentage of unbound cytochrome c relative to control samples lacking bacteria. Data represent the mean±SD of three independent experiments. (**D and E**) Flow cytometry analysis of total *Lm*-associated CRAMP levels in mid-exponential-phase *Lm* strains, following incubation (5 min) with a 5-µg/ml solution of fluorescently labeled peptide (5-FAM-CRAMP). (D) Representative experiment showing overlaid histograms of FAM-CRAMP-treated (solid line) and untreated (dashed line) samples, with MFI values from treated samples indicated by vertical dashed lines. (E) Mean±SD of the MFI values of 5-FAM-CRAMP-treated samples from three independent experiments. (**F**) Fluorometric quantification of the unbound CRAMP fraction in the supernatant of suspensions of mid-exponential-phase *Lm* strains, following incubation (5 min) with a 5-µg/ml solution of 5-FAM-CRAMP. Data are expressed as the percentage of unbound fluorescent peptide relative to control samples lacking bacteria, and represent the mean±SD of three independent experiments performed in triplicates. ns=not significant, $p>0.05$; **, $p\leq0.01$; ***, $p\leq0.001$.

respective wild type strain, as illustrated by a decreased percentage of unbound cytochrome c (Fig. 22C). However, no significant difference in cytochrome c binding levels was observed between Δ*rmlACBD*, Δ*rmlT* and wild type EGD-e strains (Fig. 22C), indicating that the absence of L-rhamnose in WTAs does not affect the *Lm* surface charge. This was further corroborated by zeta potential

measurements showing similar pH-dependent variations for both wild type and mutant strains (Fig. S5). Overall, these results allowed us to discard electrostatic changes as a reason behind the difference in CRAMP levels detected at the *Lm* cell surface.

To further explore the decreased levels of surface-exposed CRAMP in *Lm* strains lacking L-rhamnosylated WTAs, we compared total levels of bacterium-associated CRAMP in the different strains by flow cytometry, following a short incubation with a fluorescently labeled form of this AMP. The intensity of *Lm*-associated CRAMP fluorescence was comparable for the wild type EGD-e, Δ*rmlACBD* and Δ*rmlT* strains (Figs. 22D and 22E), indicating that the overall peptide levels associated to *Lm* cells were similar between the different strains. Accordingly, the residual fluorescence in the supernatants obtained by centrifugation of the bacteria-peptide suspensions was also similar (Fig. 22F). As positive control we used the Δ*dltA* strain, which displayed a significantly stronger peptide binding than its parental wild type strain (Figs. 22D–F). These data strongly suggest that the increased CRAMP susceptibility of *Lm* strains lacking L-rhamnosylated WTAs results from an improved penetration of CRAMP through their cell walls.

Altogether, these results showed that L-rhamnosylated WTAs do not interfere with the *Lm* surface charge or with the binding efficiency of AMPs, but likely promote *Lm* survival by hindering the cell wall crossing by these bactericides.

I.4.5. WTA L-rhamnosylation delays AMP interaction with the *Lm* plasma membrane

In light of these results, we then examined whether WTA L-rhamnosylation interfered with the dynamics of AMP interaction with the *Lm* plasma membrane. We performed a time-course study to follow *Lm* membrane potential changes induced by CRAMP. In live bacteria, the membrane potential is an electric potential generated across the plasma membrane by the concentration gradients of sodium, potassium and chloride ions. Physical or chemical disruption of the plasma membrane integrity leads to the suppression of this potential (depolarization) (Shapiro 2000). *Lm* strains were incubated with $DiOC_2(3)$, a green

fluorescent voltage-sensitive dye that readily enters into bacterial cells. As the intracellular dye concentration increases with higher membrane potential, it favors the formation of dye aggregates that shift the fluorescence emission to red. After stabilization of the $DiOC_2(3)$ fluorescence, CRAMP was added to bacterial samples and the rate of *Lm* depolarization was immediately analyzed by measuring the red fluorescence emission decline in a flow cytometer. The decrease in the membrane potential was consistently greater in the Δ*rmlACBD* and Δ*rmlT* strains as compared to wild type *Lm*, particularly in the first 10-15 min (Fig. 23A), indicating that the *Lm* plasma membrane integrity is compromised faster by the action of CRAMP in the absence of L-rhamnosylated WTAs. To investigate if increased CRAMP-mediated disruption of the *Lm* membrane integrity was associated with increased permeabilization, we monitored in real time the entry of the fluorescent probe SYTOX Green into the different *Lm* strains, following the addition of CRAMP. This probe only enters into bacterial cells with a compromised membrane and displays a strong green fluorescence emission after binding to nucleic acids. As expected, when CRAMP was omitted from the bacterial suspensions, any increase in SYTOX Green-associated fluorescence was detected (Fig. 23B). However, in the presence of the peptide, the green fluorescence intensity of samples containing the Δ*rmlACBD* or Δ*rmlT* mutants increased earlier than in samples containing wild type *Lm* (Fig. 23B), eventually reaching similar steady-state levels at later time points (Fig. S7). These observations indicate that CRAMP-mediated increase of the *Lm* membrane permeability occurs faster in strains lacking L-rhamnosylated WTAs.

To investigate the ultrastructural localization of the peptide, we performed immunoelectron microscopy on CRAMP-treated wild type and Δ*rmlACBD* *Lm* strains. Interestingly, CRAMP-specific labeling was not only detected in the *Lm* cell envelope, as expected, but also in the cytoplasm (Fig. 23C), suggesting that this AMP may additionally target components or processes inside *Lm*. Comparison of the subcellular distribution of CRAMP between these two bacterial compartments revealed a preferential cell envelope localization in wild type *Lm*, which contrasted with the slight but significantly higher cytoplasmic localization of the peptide in the Δ*rmlACBD* strain (Fig. 23D). These observations are in agreement with a model in which CRAMP crosses the *Lm* cell wall more efficiently in the absence of WTA L-

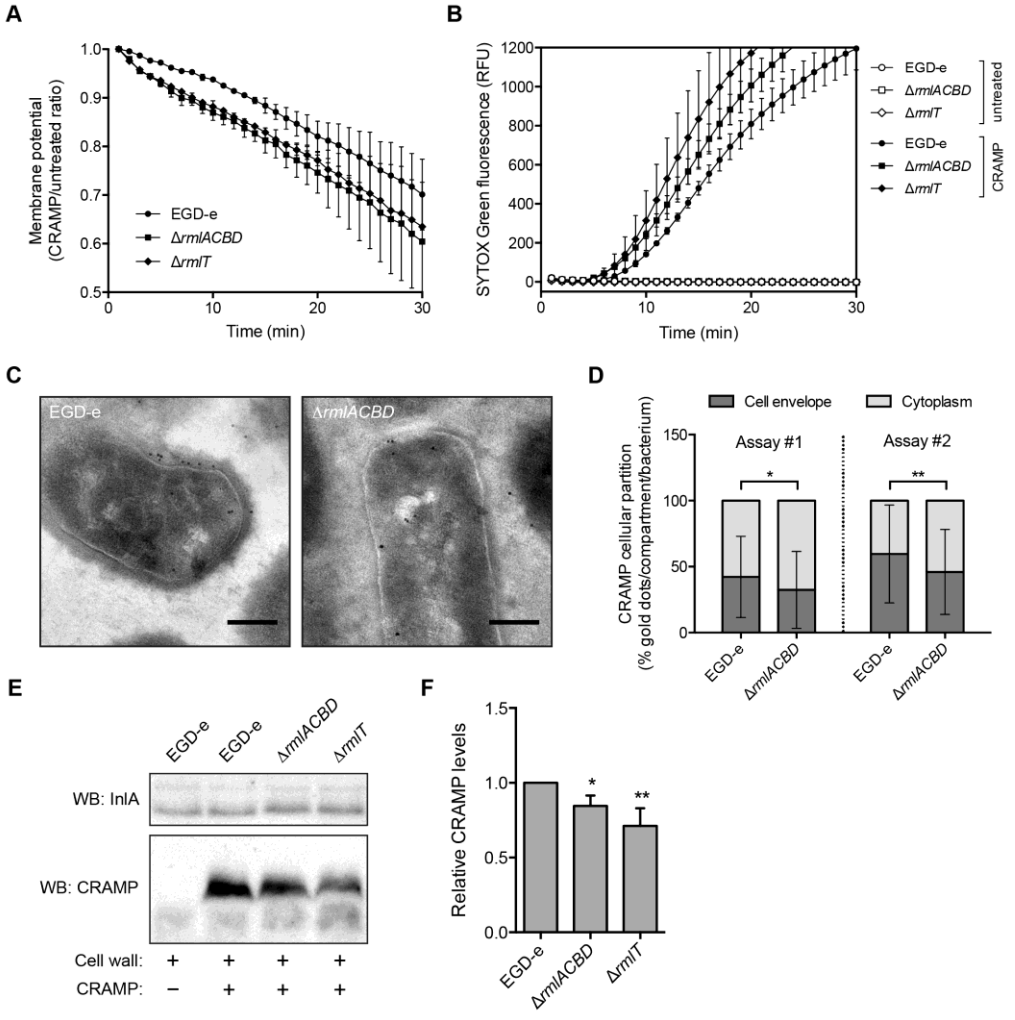

Fig. 23. **WTA** L-**rhamnosylation delays AMP interaction with the *Lm* plasma membrane.** (**A**) Depolarization rate of *Lm* strains in response to CRAMP. Mid-exponential-phase bacteria pre-stained (15 min) with 30 µM $DiOC_2(3)$ were challenged with 50 µg/ml CRAMP and changes in the membrane potential, expressed as the ratio of CRAMP-treated versus untreated samples, were monitored during 30 min. Data represent the mean±SD of three independent experiments. (**B**) SYTOX Green uptake kinetics of *Lm* strains in response to CRAMP-mediated membrane permeabilization. Exponential-phase bacteria were incubated (37 ºC) with PBS (white symbols) or 50 µg/ml CRAMP (black symbols), in the presence of 1 µM SYTOX Green, and the increase in green fluorescence emission was recorded over time. (**C and D**) Transmission electron microscopy analysis of the subcellular distribution of CRAMP in immunogold-labeled sections of mid-exponential-phase wild type and Δ*rmlACBD Lm* strains treated with 50 µg/ml CRAMP (15 min, 37 ºC). (C) Representative images of contrasted sections of *Lm* cells showing CRAMP-specific gold labeling (10-nm black dots). Scale bar: 0.2 µm. (D) Quantification of the subcellular partition of CRAMP labeling in wild type and Δ*rmlACBD Lm* strains, for two independent assays. The percentages of cell envelope- and cytoplasm-associated gold dots per bacterium were quantified (at least 90 cells per strain) and the results expressed for each strain as mean±SD. (**E and F**) Western blot analysis of levels of CRAMP bound to purified cell wall of different *Lm* strains. Purified cell wall (100 µg) was incubated with CRAMP (5 min), washed and digested overnight with mutanolysin. (E) Supernatants from mutanolysin-treated samples were resolved in 16% Tris-tricine SDS-PAGE and immunoblotted for CRAMP. The *Lm* cell wall-anchored protein InlA was used as loading control. (**F**) Quantification of the relative CRAMP levels represented as the mean±SD of four independent blots. *, $p \leq 0.05$; **, $p \leq 0.01$.

rhamnosylation, therefore reaching the bacterial membrane and the cytoplasm comparatively faster.

Finally, to confirm that the presence of L-rhamnosylated WTAs hinders the capacity of AMPs to flow through the *Lm* cell wall, we assessed levels of CRAMP retained in purified cell wall samples from the wild type, Δ*rmlACBD* and Δ*rmlT* strains by Western blot. After incubation with CRAMP, peptides trapped within the peptidoglycan matrix were released by mutanolysin treatment of the cell wall and quantitatively resolved by SDS-PAGE. Immunoblotting revealed a small but consistent decrease in the amount of peptide associated with the cell wall from the two mutant strains in comparison with wild type *Lm* (Figs. 23E and 23F). This result indicates that the lack of L-rhamnose in WTAs results in a partial loss of the AMP retention capacity of the *Lm* cell wall, which induces an enhanced AMP targeting of the *Lm* plasma membrane and consequent bacterial killing.

All combined, these data support a model where the L-rhamnosylation of WTAs alters the *Lm* cell wall permeability to favor the entrapment of AMPs. This obstructive effect hinders AMP progression through the cell wall and delays their lethal interaction with the plasma membrane.

I.4.6. WTA L-rhamnosylation is crucial for AMP resistance *in vivo* and *Lm* virulence

To evaluate the importance of WTA L-rhamnosylation in *Lm* pathogenicity, we assessed the *in vivo* virulence of *Lm* strains lacking L-rhamnosylated WTAs. BALB/c mice were inoculated orally with wild type, Δ*rmlACBD* or Δ*rmlT* strains, and the bacterial load in the spleen and liver of each animal was quantified three days later. The proliferative capacity of both Δ*rmlACBD* and Δ*rmlT* mutant strains was similarly reduced in both organs, although more significantly in the liver (Figs. 24A and 24B). To determine if the decreased virulence of the mutant strains was due to a specific defect in the crossing of the intestinal epithelium, BALB/c mice were challenged intravenously, bypassing the intestinal barrier. Three days post-infection, the differences between mutant and wild type strains, in both organs, were similar to those observed in orally infected animals (Figs. 24C and 24D), thus discarding any sieving effect of the intestinal epithelium on the decreased splenic and hepatic colonization by both Δ*rmlACBD* and Δ*rmlT*. Importantly, organs of mice infected intravenously with the complemented strains (Δ*rmlACBD*+*rmlACBD*

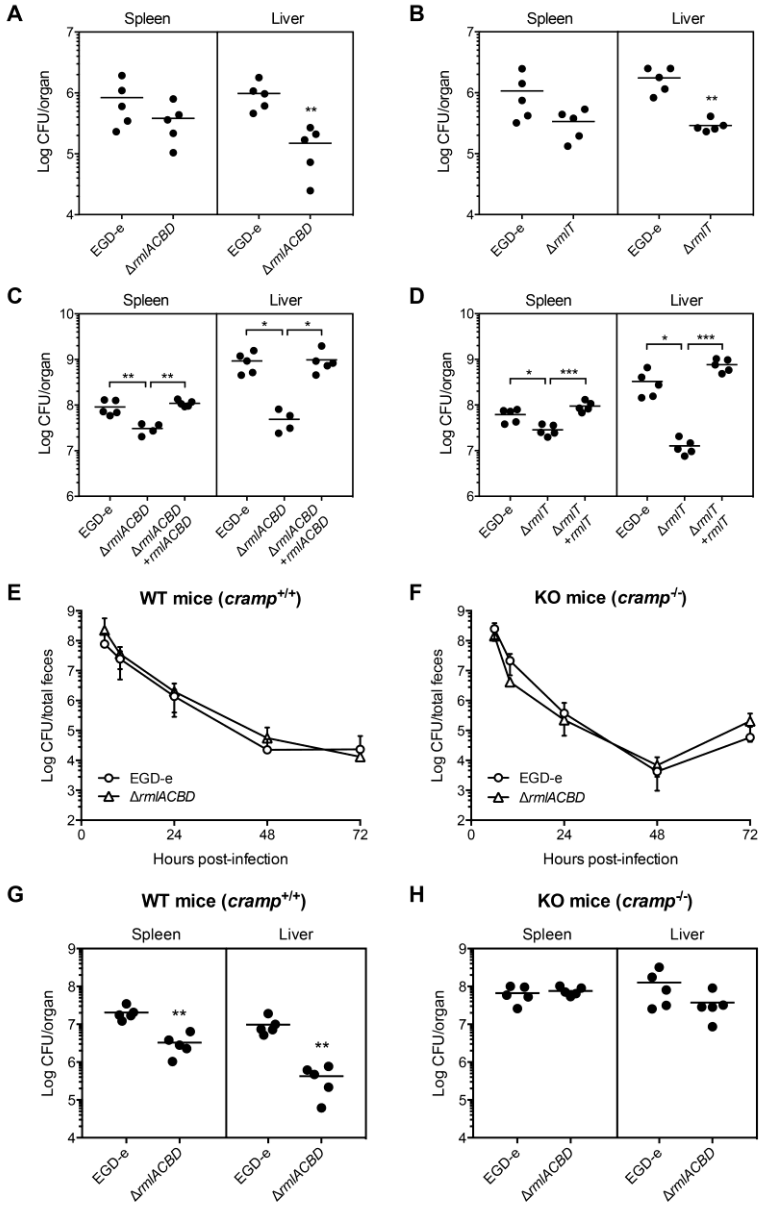

Fig. 24. **WTA L-rhamnosylation is necessary for AMP resistance *in vivo* and *Lm* virulence.** (**A–D**) Quantification of viable bacteria in the spleen and liver recovered from BALB/c mice (n=5), three days after (A and B) oral or (C and D) intravenous infection with sub-lethal doses of indicated *Lm* strains. Data are presented as scatter plots, with each animal indicated by a dot and the mean indicated by a horizontal line. **(E and F**) Quantification of the fecal shedding of wild type or Δ*rmlACBD Lm* strains after oral infection of (E) wild type (WT, *cramp*$^{+/+}$) and (F) CRAMP knockout (KO, *cramp*$^{-/-}$) 129/SvJ mice (n=5). Total feces produced by each animal at specific time points were collected and processed for bacterial enumeration in *Listeria*-selective agar media. Data are expressed as mean±SD. (**G and H**) Quantification of viable bacteria in spleens and livers recovered from (G) wild type (WT, *cramp*$^{+/+}$) and (H) CRAMP knockout (KO, *cramp*$^{-/-}$) 129/Sv mice (n=5), three days after intravenous infection with sub-lethal doses of wild type or Δ*rmlACBD Lm* strains. Data are presented as scatter plots, with each animal represented by a dot and the mean indicated by a horizontal line. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

and Δ*rmlT*+*rmlT*) displayed bacterial loads comparable to wild type *Lm*-infected organs (Figs. 24C and 24D). The attenuated *in vivo* phenotype of the Δ*rmlACBD* and Δ*rmlT* strains was not caused by an intrinsic growth defect, as demonstrated

by their wild type-like growth profiles in broth or inside eukaryotic cells (Fig. S8). These results confirmed the involvement of the *rml* operon in virulence, revealing a significant contribution of WTA L-rhamnosylation to *Lm* pathogenesis. Importantly, the *in vivo* attenuation of the Δ*rmlT* strain, which is unable to append L-rhamnose to its WTAs but is able to synthesize the L-rhamnose precursor, showed that although L-rhamnose biosynthesis is required to achieve optimal levels of virulence it is its covalent linkage to the WTA backbone that is crucial for the successful *Lm* host infection.

To evaluate the protective role of WTA L-rhamnosylation against AMPs *in vivo*, we performed virulence studies in a CRAMP-deficient mouse model. To determine the influence of WTA L-rhamnosylation in *Lm* intestinal persistence, we performed oral infections of adult CRAMP knockout 129/SvJ mice (*cramp*$^{-/-}$, KO) (Nizet *et al.* 2001) and of age- and background-matched wild type mice (*cramp*$^{+/+}$, WT), with the wild type or Δ*rmlACBD Lm* strains and monitored the respective fecal carriage. In both WT and KO mice, we observed comparable dynamics of fecal shedding of the wild type and Δ*rmlACBD* strains (Figs. 6E and 6F). In agreement with the comparable virulence defects observed for WTA L-rhamnosylation-deficient bacteria, following oral or intravenous inoculation of BALB/c mice (Figs. 24A–D), these results suggest a minor role for CRAMP in the control of *Lm* during the intestinal phase of the infection.

We then inoculated WT and KO mice intravenously and quantified bacterial numbers in the spleen and liver, three days post-infection. In line with what was observed in BALB/c mice (Fig. 6C), the Δ*rmlACBD* strain showed significant virulence attenuation in both organs of WT mice (Fig. 24G). Interestingly, this virulence defect was nearly abolished in KO animals, with the Δ*rmlACBD* strain displaying an organ-colonizing capacity similar to wild type bacteria (Fig. 6H). In addition, bacterial loads were higher in the organs of KO mice than in those of WT animals (Figs. 24G and 24H). These data indicate that, in comparison to their WT congeners, KO mice are more susceptible to *Lm* infection, and confirm the *in vivo* listericidal activity of CRAMP.

Altogether, these results highlight a key role for host-produced CRAMP in restraining *Lm* infection and demonstrate that WTA L-rhamnosylation also promotes resistance to AMPs in an *in vivo* context.

I.5. Discussion

Teichoic acids are key players in the maintenance of the Gram-positive cell envelope integrity and functionality. They are typically decorated with D-alanine and/or a variety of glycosyl groups, which influence the overall properties of these polymers (Weidenmaier and Peschel 2008). Whereas D-alanylation of WTAs has been demonstrated to contribute towards bacterial defense against AMPs (Peschel *et al.* 1999, Koprivnjak *et al.* 2002), the involvement of glycosylation in this process has never been investigated. In this study, we show for the first time that the glycosylation of *Lm* WTAs with L-rhamnose is mediated by the WTA L-rhamnosyltransferase RmlT and confers protection against AMPs *in vitro* and during mouse infection. Based on our data, we propose that this protection results from a delayed traversal of the *Lm* cell envelope by AMPs in the presence of L-rhamnose-decorated WTAs. Most importantly, we reveal a key role for L-rhamnosylated WTAs in the processes underlying *Lm* pathogenesis.

Unlike *S. aureus* or *B. subtilis* (Neuhaus and Baddiley 2003), WTAs in *Listeria* are not decorated with D-alanine, undergoing only glycosylation with a small pool of monosaccharides (Uchikawa *et al.* 1986a, Fiedler 1988). Among these is L-rhamnose, which is the product of a remarkably conserved biosynthetic pathway that is encoded by the *rmlABCD* genes (Giraud and Naismith 2000). Interestingly, a significant number of bacteria harboring these genes are commonly pathogenic (Macpherson *et al.* 1994, Tsukioka *et al.* 1997, Li and Reeves 2000, Li *et al.* 2003, Li *et al.* 2006, Aguirre-Ramírez *et al.* 2012) and have L-rhamnose in close association with surface components (Chatterjee 1997, Frirdich and Whitfield 2005). In *Listeria*, the *rmlACBD* locus is only found in certain serotypes of *Lm* (1/2a, 1/2b, 1/2c, 3c and 7) and *L. seeligeri* (1/2b). These serotypes were all shown to have L-rhamnose in their WTAs, except for *Lm* serotypes 3c and 7 (Fiedler 1988), which appear to be unable to produce this sugar because of mutations within *rmlA* and *rmlB*, respectively (Fig. 19). Our results confirmed that the appendage of L-rhamnose to *Lm* WTAs requires the products of the *rmlACBD* locus. Ultimately, WTA glycosylation is catalyzed by glycosyltransferases, a class of enzymes that recognize nucleotide-sugar substrates and transfer the glycosyl moiety to a WTA subunit (Lairson *et al.* 2008). *In silico* analysis of *lmo1080*, the first gene of the operon including *rmlACBD*

(Fig. 19) showed that it encodes a protein with putative glycosyltransferase activity. The genomic location and predicted protein function were strong indicators that this gene might encode the transferase involved in the L-rhamnosylation of *Lm* WTAs. Our data demonstrated that whereas *lmo1080*, that we renamed *rmlT*, is dispensable for rhamnose biosynthesis, it is required for the addition of L-rhamnose to WTAs in *Lm* strains with a functional L-rhamnose pathway, thus validating RmlT as the L-rhamnose-specific WTA glycosyltransferase in *Lm*.

WTAs are associated with the natural resistance of *S. aureus* to peptidoglycan-degrading enzymes, such as lysozyme (Bera *et al.* 2007, Atilano *et al.* 2010). In contrast, absence of WTA decoration, but not of the polymers, was shown to induce an increase of the staphylococcal susceptibility to lysostaphin (Brown *et al.* 2012). Modifications of the *Lm* peptidoglycan, such as *N*-deacetylation (Boneca *et al.* 2007), were found to contribute to protection against lysozyme, but the role of WTAs and in particular their decoration, was never addressed. Our results discard WTA L-rhamnosylation as a component of the *Lm* resistance mechanism to this host immune defense protein, as well as its involvement in the promotion of growth under osmotic conditions. Other innate immune effectors, such as antimicrobial peptides (AMPs), also target bacterial organisms (Guilhelmelli *et al.* 2013) that in turn have developed resistance strategies to avoid injury and killing induced by AMPs. Among these strategies is the reshaping and fine-tuning of cell envelope components to lower AMP affinity to the bacterial surface (Koprivnjak and Peschel 2011). Previous studies showed a clear link between the D-alanylation of WTAs and AMP resistance (Peschel *et al.* 1999, Kovacs *et al.* 2006). In this context, we found here a similar role for WTA L-rhamnosylation, showing that, in the absence of L-rhamnosylated WTAs, bacteria exhibit an increased susceptibility to AMPs produced by bacteria, mice and importantly by humans. Although from such distinct sources, AMPs used here share a cationic nature that supports their activity. However, while teichoic acid D-alanylation is known to reduce the cell wall electronegativity (Peschel *et al.* 1999), glycosyl substituents of *Lm* WTAs are neutrally charged and WTA glycosylation should thus promote AMP resistance through a different mechanism.

It is well established that AMPs induce bacterial death mainly by tampering with the integrity of the plasma membrane. This can be achieved through multiple ways, all of which are driven by the intrinsic amphipathic properties of this class of peptides (Nguyen *et al.* 2011). Nonetheless, the initial interaction of AMPs with bacterial surfaces is mediated by electrostatic forces between their positive net charge and the anionic cell envelope (Koprivnjak *et al.* 2002). Our data show that, unlike D-alanylation (Vadyvaloo *et al.* 2004), WTA L-rhamnosylation does not interfere with the *Lm* cell surface charge, in agreement with L-rhamnose being an electrostatically neutral monosaccharide. Importantly, the reduced levels of surface-exposed CRAMP in *Lm* strains lacking L-rhamnosylated WTAs suggested instead that their increased susceptibility to this peptide was correlated with its improved penetration of the L-rhamnose-depleted *Lm* cell wall. We confirmed this premise with data showing that CRAMP-mediated cell depolarization and plasma membrane permeabilization events occur earlier in WTA L-rhamnosylation-deficient *Lm* strains. In addition, we also observed a predominant cytoplasmic presence of CRAMP in these mutant strains, in contrast to the preferential cell envelope localization in wild type *Lm*, further suggesting a WTA L-rhamnosylation-dependent kinetic discrepancy in the progression of CRAMP through the *Lm* cell envelope. Saar-Dover *et al.* demonstrated in the WTA-lacking *Streptococcus agalactiae* (GBS) that LTA D-alanylation promoted resistance to the human cathelicidin LL-37 by hindering cell wall crossing and plasma membrane disturbance (Saar-Dover *et al.* 2012). They proposed that the underlying mechanism does not rely on modulation of the surface charge but on LTA conformation-associated alterations of the cell wall packing density (Saar-Dover *et al.* 2012). Our data are in line with these observations and although we did not detect changes in the cell wall cross-linking status, we cannot ignore a possible impact of L-rhamnosylation on WTA polymer conformation accounting for changes in cell wall permeability. If one considers that the peptidoglycan, a multi-layered and compact structure, is densely populated with WTA polymers decorated with multiple units of the rather bulky L-rhamnose molecule, spatial constraints and increased cell wall density need to be accounted. In fact, we showed that purified *Lm* cell wall depleted of L-rhamnose does not retain CRAMP in its peptidoglycan matrix as effectively as cell wall containing L-rhamnosylated WTAs. In addition, we

have indications that soluble L-rhamnose interferes with CRAMP activity, improving the survival of WTA L-rhamnosylation mutants of *Lm* (data not shown). These observations suggest a potential interaction between L-rhamnose and AMPs, which could favor the “retardation effect” that ultimately promotes *Lm* survival.

We previously reported a significantly increased transcription of *rmlACBD* during mouse spleen infection (Camejo *et al.* 2009), which suggested that WTA L-rhamnosylation is highly activated by *Lm* to successfully infect this host organ. Our infection studies in mice confirmed the importance of this mechanism for *Lm* pathogenesis by revealing a significant virulence attenuation of WTA L-rhamnosylation-deficient *Lm* strains. Surprisingly, the expression of *rmlT* appeared unchanged during mouse spleen infection as compared to growth in BHI (Camejo *et al.* 2009), suggesting that an increased L-rhamnose biosynthesis could be sufficient to induce an increased WTA L-rhamnosylation and AMP resistance. Faith *et al.* also observed a decreased bacterial burden of a serotype 4b *Lm* strain lacking the *gtcA* gene (Faith *et al.* 2009), a mutation that resulted in complete loss of galactose decoration of its WTAs (Promadej *et al.* 1999). Interestingly, *gtcA* is also present in *Lm* EGD-e, where it appears to be involved in WTA substitution with *N*-acetylglucosamine (Eugster *et al.* 2011), and was shown to contribute to the colonization of the mouse spleen, liver and brain (Autret *et al.* 2001). However the mechanism through which this occurs remains unclear.

Virulence studies in mice lacking the CRAMP gene corroborated our *in vitro* susceptibility data and revealed the importance of WTA L-rhamnosylation-promoted resistance to AMPs for *Listeria* virulence. *In vivo* data also provided a strong insight into the protective role of CRAMP against systemic infection by *Lm*, as had been previously observed with other bacterial pathogens (Nizet *et al.* 2001, Huang *et al.* 2007, Chromek *et al.* 2012). Our results on fecal shedding dynamics suggest that the contribution of CRAMP to the control of *Lm* during the intestinal phase of infection is minimal. A previous report showed a negligible enteric secretion of CRAMP in normal adult mice (Ménard *et al.* 2008), which may explain the similar shedding behavior of the wild type and Δ*rmlACBD* strains that were observed in both mouse strains. In this scenario, infection studies in newborn animals, whose enterocytes actively express CRAMP (Gallo *et al.* 1997, Ménard *et*

al. 2008), may provide conclusive information regarding the role of WTA L-rhamnosylation in the *Lm* resistance to CRAMP during the intestinal phase of the infection. Notwithstanding, CRAMP is actively produced by phagocytes in adult mice (Rosenberger *et al.* 2004). As a major target for *Lm* colonization, the spleen is also an important reservoir of phagocytic cells. We can speculate that WTA L-rhamnosylation is particularly important to increase the chances of *Lm* surviving CRAMP-mediated killing during spleen infection. Considering our data on the *Lm* susceptibility to LL-37, the human homolog of CRAMP, we can also envisage this scenario in the context of human infection.

In conclusion, our work has unveiled for the first time a role for WTA glycosylation in bacterial resistance to AMPs. We propose that WTA L-rhamnosylation reduces the cell wall permeability to AMPs, promoting a delay in the crossing of this barrier and in the disruption of the plasma membrane, thus favoring *Lm* survival and virulence *in vivo*. Our findings reveal a novel facet in the contribution of WTA modifications towards AMP resistance, reinforcing the crucial role of these Gram-positive surface glycopolymers in host defense evasion.

I.6. Materials and Methods

I.6.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *Lm* and *E. coli* strains were routinely cultured aerobically at 37 ºC in brain heart infusion (BHI, Difco) and Lysogeny Broth (LB) media, respectively, with shaking. For experiments involving the *Lm* Δ*tagO1*Δ*tagO2* strain, bacteria were first cultured overnight at 30 ºC with shaking in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), washed and diluted (1:100) in fresh BHI and cultured overnight at 30 ºC with shaking (Eugster and Loessner 2012). When appropriate, the following antibiotics were included in culture media as selective agents: ampicilin (Amp), 100 µg/ml; chloramphenicol (Cm), 7 µg/ml (*Lm*) or 20 µg/ml (*E. coli*); erythromycin (Ery), 5 µg/ml. For genetic complementation purposes, colistin sulfate (Col) and nalidixic acid (Nax) were used at 10 and 50 µg/ml, respectively.

I.6.2. Construction and complementation of mutant strains

Lm mutant strains were constructed in the EGD-e background through a process of double homologous recombination mediated by the suicide plasmid pMAD (Arnaud *et al.* 2004). DNA fragments corresponding to the 5'- and 3'-flanking regions of the *rmlACBD* locus (*lmo1081–4*) were amplified by PCR from *Lm* EGD-e chromosomal DNA with primers 1–2 and 3–4 (Table S2), and cloned between the *Sal*I–*Mlu*I and *Mlu*I–*Bgl*II sites of pMAD, yielding pDC303. Similarly, DNA fragments corresponding to the 5'- and 3'-flanking regions of *rmlT* (*lmo1080*) were amplified with primers 15–16 and 17–18 (Table S2), and cloned between the *Sal*I–*Eco*RI and *Eco*RI–*Bgl*II sites of pMAD, yielding pDC491. The plasmid constructs were introduced in *Lm* EGD-e by electroporation and transformants selected at 30 ºC in BHI–Ery. Positive clones were re-isolated in the same medium and grown overnight at 43 ºC. Integrant clones were inoculated in BHI broth and grown overnight at 30 ºC, after which the cultures were serially diluted, plated in BHI agar and incubated overnight at 37 ºC. Individual colonies were tested for growth in BHI–Ery at 30 ºC and antibiotic-sensitive clones were screened by PCR

for deletion of *rmlACBD* (primers 5–6, 7–8, 9–10 and 11–12) and *rmlT* (primers 19–20) (Table S2). Genetic complementation of the deletion mutant strains was performed as described (Camejo *et al.* 2009). DNA fragments containing either the *rmlACBD* or *rmlT* loci were amplified from *Lm* EGD-e chromosomal DNA with primers 13–14 and 21–22 (Table S2), respectively, and cloned between the *Sal*I–*Pst*I sites of the phage-derived integrative plasmid pPL2 (Lauer *et al.* 2002), generating pDC313 and pDC550. The plasmid constructs were introduced in the *E. coli* strain S17-1 and transferred, respectively, to the Δ*rmlACBD* and Δ*rmlT* strains by conjugation on BHI agar. Transconjugant clones were selected in BHI–Cm/Col/Nax and chromosomal integration of the plasmids confirmed by PCR with primers 23 and 24 (Table S2). All plasmid constructs and gene deletions were confirmed by DNA sequencing.

I.6.3. Gene expression analyses

Total bacterial RNA was isolated from 10 ml of exponential cultures (OD_{600}=0.6) by the phenol-chloroform extraction method, as previously described (Milohanic *et al.* 2003), and treated with DNase I (Turbo DNA-free, Ambion), as recommended by the manufacturer. Purified RNAs (1 µg) were reverse-transcribed with random hexamers, using iScript cDNA Synthesis kit (Bio-Rad Laboratories). Quantitative real-time PCR (qPCR) was performed in 20-µl reactions containing 2 µl of cDNA, 10 µl of SYBR Green Supermix (Bio-Rad Laboratories) and 0.25 µM of forward and reverse primers (Table S2), using the following cycling protocol: 1 cycle at 95 ºC (3 min) and 40 cycles at 95 ºC (30 s), 55 ºC (30 s) and 72 ºC (30 s). Each target gene was analyzed in triplicate and blank (water) and DNA contamination controls (unconverted DNase I-treated RNA) were included for each primer pair. Amplification data were analyzed by the comparative threshold (ΔΔCt) method, after normalization of the test and control sample expression values to a housekeeping gene (16S rRNA). For qualitative analysis, PCR was performed in 20-µl reactions containing 2 µl of cDNA, 10 µl of MangoMix 2× reaction mix (Bioline) and 0.5 µM of forward and reverse qPCR primers, using the following protocol: 1 cycle at 95 ºC (5 min), 25 cycles at 95 ºC (30 s), 55 ºC (30 s) and 72 ºC (20 s), and 1 cycle at 72 ºC (5 min). Amplification

products were resolved in 1% (w/v) agarose gel and analyzed in a GelDoc XR+ System (Bio-Rad Laboratories).

I.6.4. WTA PAGE analysis

Extraction and analysis of *Lm* WTAs by polyacrylamide gel electrophoresis was performed essentially as described (Carvalho *et al.* 2013), with the exception that WTAs extracts were obtained from exponential-phase cultures. Sedimented bacteria were washed (buffer 1: 50 mM MES buffer, pH 6.5) and boiled for 1 h (buffer 2: 4% SDS in buffer 1). After centrifugation, the pellet was serially washed with buffer 2, buffer 3 (2% NaCl in buffer 1) and buffer 1, before treatment with 20 µg/ml proteinase K (20 mM Tris-HCl, pH 8; 0.5% SDS) at 50 ºC for 4 h. The digested samples were thoroughly washed with buffer 3 and distilled water and incubated overnight (16 h) with 0.1 M NaOH, under vigorous agitation. Cell wall debris were removed by centrifugation (10,000 rpm, 10 min) and the hydrolyzed WTAs present in the supernatant were directly analyzed by native PAGE in a Tris-tricine buffer system. WTA extracts were resolved through a vertical (20 cm) polyacrylamide (20%) gel at 20 mA for 18 h (4 ºC). To visualize WTAs, the gel was stained in 0.1% Alcian blue (40% ethanol; 5% acetic acid) for 30 min and washed (40% ethanol; 10% acetic acid) until the background is fully cleared. Optionally, for increased contrasting, silver staining can be performed on top of the Alcian blue staining.

I.6.5. Purification of cell wall components

Cell walls of *Lm* strains were purified as described before (Filipe *et al.* 2005), with modifications. Overnight cultures were subcultured into 1–2 liters of BHI broth (initial OD_{600}=0.005) and bacteria grown until exponential phase (OD_{600}=1.0–1.5). Cultures were rapidly cooled in an ice/ethanol bath and bacteria harvested by centrifugation (7,500 rpm, 15 min, 4 °C). The pellet was resuspended in cold ultrapure water and boiled for 30 min with 4% SDS to kill bacteria and inactivate cell wall-modifying enzymes. The samples were cleared of SDS by successive cycles of centrifugation (12,000 rpm, 10 min) and washing with warm ultrapure

water until no detergent was detected (Hayashi 1975). SDS-free samples were resuspended in 2 ml of ultrapure water and cell walls disrupted with glass beads in a homogenizer (FastPrep, Thermo Savant). Fully broken cell walls were separated from glass beads by filtration (glass filters, pore size: 16-40 µm) and from unbroken cell walls and other debris by low-speed centrifugation (2,000 rpm, 15 min). Nucleic acids were degraded after incubation (2 h) at 37 °C with DNase (10 µg/ml) and RNase (50 µg/ml) in a buffer containing 50 mM Tris-HCl, pH 7.0, and 20 mM $MgSO_4$. Proteins were then digested overnight at 37 °C with trypsin (100 µg/ml) in the presence of 10 mM $CaCl_2$. Nuclease and proteases were inactivated by boiling in 1% SDS, and samples were centrifuged (17,000 rpm, 15 min) and washed twice with ultrapure water. Cell walls were resuspended and incubated (37 °C, 15 min) in 8 M LiCl and then in 100 mM EDTA, pH 7.0, after which they were washed twice with water. After resuspension in acetone and sonication (15 min), cell walls were washed and resuspended in ultrapure water before undergoing lyophilization.

To obtain purified peptidoglycan, cell walls (20 mg) were incubated for 48 h with 4 ml of 46% hydrofluoric acid (HF), under agitation at 4 °C. Samples were washed with 100 mM Tris-HCl, pH 7.0, and centrifuged (17,000 rpm, 30 min, 4 °C) as many times as necessary to neutralize the pH. The pellet was finally washed twice with water prior to lyophilization. WTA extracts were obtained by incubating 1 mg of cell wall with 300 µl of 46% HF (18 h, 4 °C). After centrifugation (13,200 rpm, 15 min, 4 °C), the supernatant was recovered and evaporated under a stream of compressed air. The dried WTA residue was resuspended in water and lyophilized.

I.6.6. Extraction of bacterial cytoplasmic content

The intracellular content of *Lm* strains was isolated according to a modified version of the protocol by Ornelas-Soares *et al*. (Ornelas-Soares *et al.* 1994). Bacterial cultures (200 ml) were grown until early exponential phase (OD_{600}=0.3), and vancomycin was added at 7.5 µg/ml (5×MIC value (Blanot *et al.* 1999)) to induce the cytoplasmic accumulation of the peptidoglycan precursor UDP-MurNAc-pentapeptide. Cultures were grown for another 45 min and chilled in an

ice-ethanol bath for 10 min. Bacteria were then harvested by centrifugation (12,000 rpm, 10 min, 4 ºC), washed with cold 0.9% NaCl, resuspended in 5 ml of cold 5% trichloroacetic acid (TCA) and incubated for 30 min on ice. Cells and other debris were separated by centrifugation (4,000 rpm, 15 min, 4 ºC) and the supernatant was extracted with 1-2 volumes of diethyl ether as many times as necessary to remove TCA (sample pH should rise to at least 6.0). The aqueous fraction containing the cytoplasmic material was lyophilized and the dried residue resuspended in ultrapure water.

I.6.7. HPLC analyses

To analyze their sugar composition, purified cell wall and peptidoglycan (200 µg each), as well as cytoplasmic (500 µg) and WTA extracts were hydrolyzed in 3 M HCl for 2 h at 95 °C. After vacuum evaporation, the samples were washed with water and lyophilized. The hydrolyzed material was then resuspended in 150 µl of water and resolved by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). Ten microliters were injected into a CarboPac PA10 column (Dionex, Thermo Fisher Scientific) and eluted at 1 ml/min (30 °C) with 18 mM NaOH, followed by a gradient of $NaCH_3COO$: 0–20 mM (t=25–30 min), 20–80 mM (t=30–35 min), 80–0 mM (t=40–45 min). Standards for glucosamine, muramic acid, L-rhamnose and ribitol (Sigma-Aldrich) were eluted under the same conditions to enable identification of chromatogram peaks. Data were acquired and analyzed with the Chromeleon software (Dionex, Thermo Fisher Scientific).

Muropeptide samples were prepared and analyzed as described (de Jonge *et al.* 1992), with minor changes. Purified peptidoglycan was digested with 200 µg/ml mutanolysin (Sigma-Aldrich) in 12.5 mM sodium phosphate, pH 5.5, for 16 h at 37 °C. Enzymatic activity was halted by heating at 100 °C for 5 min, after which the digested sample was reduced for 2 h with 2.5 mg/ml of sodium borohydride ($NaBH_4$) in 0.25 M borate buffer, pH 9.0. The reaction was stopped by lowering the sample pH to 2 with ortho-phosphoric acid. After centrifugation, the supernatant was analyzed by reverse phase HPLC. Fifty microliters were injected into a Hypersil ODS (C18) column (Thermo Fisher Scientific) and muropeptide species

eluted (0.5 ml/min, 52 °C) in 0.1 M sodium phosphate, pH 2.0, with a gradient of 5–30% methanol and detected at 206 nm.

I.6.8. Intracellular multiplication

Mouse macrophage-like J774A.1 cells (ATCC, TIB-67) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and infection assays were performed as described (Camejo *et al.* 2009). Briefly, cells (~2×10^5/well) were infected for 45 min with exponential-phase bacteria at a multiplicity of infection of ~10 and treated afterwards with 20 µg/ml gentamicin for 75 min. At several time-points post-infection, cells were washed with PBS and lysed in cold 0.2% Triton X-100 for quantification of viable intracellular bacteria in BHI agar. One experiment was performed with triplicates for each strain and time-point.

I.6.9. Resistance to salt stress and lysozyme

Lm cultures grown overnight were appropriately diluted in BHI broth and their growth under the presence of stressful stimuli was monitored by optical density measurement at 600 nm (OD_{600}). For comparative analysis of *Lm* resistance to salt stress, bacterial cultures were diluted 100-fold in BHI alone (control) or BHI containing 5% NaCl. To assess the *Lm* resistance to lysozyme, exponential-phase cultures ($OD_{600} \approx 1.0$) were challenged with different doses of chicken egg white lysozyme (Sigma). A mutant *Lm* strain hypersensitive to lysozyme (Δ*pgdA*) was used as a positive control for susceptibility.

I.6.10. AMP susceptibility

Bacteria in the exponential phase of growth (OD_{600}=0.7–0.8) were diluted (10^4 CFU/ml) in sterile PB medium (10 mM phosphate buffer, pH 7.4; 1% BHI) and mixed in a 96-well microplate with increasing concentrations of gallidermin (Santa Cruz Biotechnology), CRAMP or LL-37 (AnaSpec). Bacterial suspensions without AMPs were used as reference controls for optimal growth/survival. After incubation

for 2 h at 37 ºC, the mixtures were serially diluted in sterile PBS and plated in BHI agar for quantification of viable bacteria. Each condition was analyzed in duplicate in three independent assays.

I.6.11. Cytochrome c binding

Cytochrome c binding assays were performed as described (Vadyvaloo *et al.* 2004). Bacteria from mid-exponential-phase cultures (OD_{600}=0.6–0.7) were washed in 20 mM MOPS buffer, pH 7.0, and resuspended in ½ volume of 0.5 mg/ml equine cytochrome c (Sigma-Aldrich) in 20 mM MOPS buffer, pH 7.0. After 10 min of incubation, bacteria were pelleted and the supernatant collected for quantification of the absorbance at 530 nm. The mean absorbance values from replicate samples containing bacteria were subtracted to the mean value of a reference sample lacking bacteria, and the results were presented for each strain as percentage of unbound cytochrome c.

I.6.12. Zeta potential measurements

Bacteria (1 ml) from mid-exponential-phase cultures were washed twice with deionized water and diluted (10^7 CFU/ml) in 15 mM NaCl solutions adjusted to different pH values (1 to 7) with nitric acid. Bacterial suspensions (750 µl) were injected into a disposable capillary cell cuvette (DTS1061, Malvern Instruments) and the zeta potential was measured at 37 ºC in a ZetaSizer Nano ZS (Malvern Instruments), under an automated field voltage. Samples were measured in triplicate in three independent assays.

I.6.13. Flow cytometry analyses

Bacteria from 500 µl of mid-exponential-phase cultures were washed twice with PBS and treated for 5 min with 5 µg/ml CRAMP or PBS (untreated control). After centrifugation, the supernatant was removed and PBS-washed bacteria were incubated for 1 h with rabbit anti-CRAMP (1:100, Innovagen), followed by 1 h with Alexa Fluor 488-conjugated anti-rabbit IgG (1:200, Molecular Probes). Finally,

bacteria were fixed with 3% paraformaldehyde for 15 min, washed and resuspended in PBS. Alternatively, bacteria were similarly treated with an N-terminally 5-FAM-labeled synthetic form of CRAMP (95% purity, Innovagen), washed and resuspended in PBS. Samples were acquired in a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences) and data were analyzed with FlowJo (TreeStar Inc.). Green fluorescence was collected from at least 50,000 FSC/SSC-gated bacterial events in the FL1 channel (530 nm/20 nm bandpass filter). Fluorescence intensities were plotted in single-parameter histograms and results were presented as the average mean fluorescence intensity (MFI) value from three independent analyses.

For bacterial membrane potential studies, the lipophilic fluorescent probe $DiOC_2(3)$ (3,3-diethyloxacarbocyanine, Santa Cruz Biotechnology) was used as a membrane potential indicator (Novo *et al.* 1999, Shapiro 2000). Mid-logarithmic phase bacteria were diluted (10^6 CFU/ml) in PBS with 30 µM $DiOC_2(3)$ and incubated for 15 min in the dark. CRAMP was added to a final concentration of 50 µg/ml and the sample was immediately injected in the flow cytometer. Control samples treated with PBS or with 1.5 mM sodium azide (uncoupling agent) were analyzed to determine the fluorescence values corresponding to basal (100%) and null (0%) membrane potential (Fig. S6). Green and red (FL3, 670 nm/long bandpass filter) fluorescence emissions were continuously collected from FSC/SSC-gated bacteria for 30 min. After acquisition, a ratio of red over green fluorescence (R/G) was calculated per event and plotted in the y-axis versus time. A series of consecutive one-minute-wide gates was applied to the plot and the mean R/G value per gate was determined. The mean R/G values from uncoupler-treated samples were deducted from the corresponding values from the untreated and CRAMP-treated samples, and the resulting values for each condition were normalized as percentage of the initial value (t=1 min). Finally, the temporal variation of the *Lm* membrane potential was represented graphically as the ratio of the normalized values from CRAMP-treated over untreated samples.

I.6.14. SYTOX Green uptake

Bacterial uptake of the cell-impermeable SYTOX Green dye was used to

study membrane permeabilization induced by CRAMP (Saar-Dover *et al.* 2012). Exponential-phase bacteria were washed and resuspended (10^7 CFU/ml) in sterile PBS containing 1 µM SYTOX Green (Molecular Probes). After 20 min of incubation in the dark, bacterial suspensions were mixed in PCR microplate wells with 50 µg/ml CRAMP or PBS (negative control) for a total volume of 100 µl. The mixtures were immediately placed at 37 ºC in a real-time PCR detection system (iQ™5, Bio-Rad Laboratories) and fluorescence emission at 530 nm was recorded every minute following excitation at 488 nm.

I.6.15. Binding of AMP to purified cell walls

One-hundred micrograms of purified cell wall were resuspended in 50 µl of 5 µg/ml CRAMP or PBS (negative control) and gently shaken for 5 min. Samples were centrifuged (16,000 × g, 1 min), washed in PBS and in TM buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4) before overnight incubation at 37 °C with mutanolysin (400 U/ml) in TM buffer (50 µl). Supernatants were resolved by tricine-SDS-PAGE in a 16% gel, transferred onto nitrocellulose membrane and blotted with rabbit anti-CRAMP (1:1000) or mouse anti-InlA (L7.7; 1:1000), followed by HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:2000, P.A.R.I.S). Immunolabeled bands were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce) and digitally acquired in a ChemiDoc XRS+ system (Bio-Rad Laboratories).

I.6.16. Immunoelectron microscopy

Exponential-phase bacteria treated with 50 µg/ml CRAMP for 15 min at 37 ºC were fixed for 1 h at room temperature (4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.2), stained with 1% osmium tetroxide for 2 h and resuspended in 30% BSA (high-purity grade). Bacterial pellets obtained after centrifugation in microhematocrit tubes were fixed overnight in 1% glutaraldehyde, dehydrated in increasing ethanol concentrations, and embedded in Epon 812. Ultrathin sections (40–50 nm) were placed on 400-mesh Formvar-coated copper grids and treated with 4% sodium metaperiodate and 1%

periodic acid (10 min each) for antigen retrieval. For immunogold labeling of CRAMP, sections were blocked for 10 min with 1% BSA and incubated overnight (4 ºC) with rabbit anti-CRAMP (1:100 in 1% BSA). After extensive washing, sections were labeled with 10-nm gold complex-conjugated anti-rabbit IgG (1:200 in 1% BSA) for 2 h, washed and contrasted with 4% uranyl acetate and 1% lead citrate. Images were acquired in a Jeol JEM-1400 transmission electron microscope equipped with a Gatan Orius SC1000 CCD camera and analyzed using ImageJ software.

I.6.17. Animal infections

Virulence studies were done in mouse models of the following strains: wild type BALB/c and 129/SvJ (Charles River Laboratories); and CRAMP-deficient (*cramp*$^{-/-}$) 129/SvJ, which was bred in our facilities from a breeding pair provided by Dr. Richard L. Gallo (University of California, USA) (Nizet *et al.* 2001). Infections were performed in six-to-eight week-old specific-pathogen-free females as described (Cabanes *et al.* 2008). Briefly, for oral infections, 12-h starved animals were inoculated by gavage with 10^9 CFU in PBS containing 150 mg/ml $CaCO_3$, while intravenous infections were performed through the tail vein with 10^4 CFU in PBS. In both cases, the infection was carried out for 72 h, at which point the animals were euthanatized by general anesthesia. The spleen and liver were aseptically collected, homogenized in sterile PBS, and serial dilutions of the organ homogenates plated in BHI agar. For analysis of *Lm* fecal carriage, total feces produced by each infected animal (n=5 per strain) up to a given time-point were collected, homogenized in PBS and serial dilutions plated in *Listeria* selective media (Oxoid) for bacterial enumeration. Mice were maintained at the IBMC animal facilities, in high efficiency particulate air (HEPA) filter-bearing cages under 12 h light cycles, and were given sterile chow and autoclaved water *ad libitum*.

I.6.18. Ethics Statement

All the animal procedures were in agreement with the guidelines of the European Commission for the handling of laboratory animals (directive

2010/63/EU), with the Portuguese legislation for the use of animals for scientific purposes (Decreto-Lei 113/2013), and were approved by the IBMC Animal Ethics Committee, as well as by the Direcção Geral de Veterinária, the Portuguese authority for animal protection, under license PTDC/SAU-MIC/111581/2009.

I.6.19. Statistical analyses

Statistical analyses were performed with Prism 6 (GraphPad Software). Unpaired two-tailed Student's *t*-test was used to compare the means of two groups; one-way ANOVA was used with Tukey's post-hoc test for pairwise comparison of means from more than two groups, or with Dunnett's post-hoc test for comparison of means relative to the mean of a control group. Mean differences were considered statistically non-significant (ns) when *p* value was above 0.05. For statistically significant differences: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

I.7. Acknowledgements

We thank Catarina Leitão from the Advanced Flow Cytometry Unit and Rui Fernandes from the Histology and Electron Microscopy Service at IBMC for their technical assistance; Pascale Cossart and Martin Loessner, for kindly providing us with the EGD Δ*dltA* and EGD-e Δ*tagO1*Δ*tagO2*::pLIV2(*tagO1*) strains, respectively; and Francisco S. Mesquita, for critical reading of the manuscript. We are also grateful to Prof. Rui Appelberg for PhD co-supervision of FC and RP.

I.8. Tables

Table 1. Plasmids and bacterial strains

Plasmid or strain	Code	Relevant characteristics	Source
Plasmids			
pMAD		Gram-negative/Gram-positive shuttle vector; thermosensitive replication; Amp^r Ery^r	Arnaud *et al.* 2004
pPL2		*L. monocytogenes* phage-derived site-specific integration vector; Cm^r	Lauer *et al.* 2002
pMAD(Δ*rmlACBD*)	pDC303	pMAD with 5'- and 3'-flanking regions of *rmlACBD* locus; Amp^r Ery^r	This study
pPL2(*rmlACBD*)	pDC313	pPL2 with *rmlACBD* locus and 5'- and 3'-flanking regions; Cm^r	This study
pMAD(Δ*rmlT*)	pDC491	pMAD with 5'- and 3'-flanking regions of *rmlT*; Amp^r Ery^r	This study
pPL2(*rmlT*)	pDC550	pPL2 with *rmlT* sequence and 5'- and 3'-flanking regions; Cm^r	This study
***E. coli* strains**			
DH5α		Cloning host strain; F^- Φ80*lac*ZΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Life Technologies
S17-1		Conjugative donor strain; *rec*A *pro hsdR* RP4-2-Tc::Mu-Km::Tn7	Simon *et al.* 1983
***L. monocytogenes* strains**			
EGD-e		wild type; serotype 1/2a	Glaser *et al.* 2001
EGD-e Δ*pgdA*		EGD-e *pgdA* (*lmo0415*) deletion mutant	Boneca *et al.* 2007
EGD-e Δ*rmlACBD*	DC307	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant	This study
EGD-e Δ*rmlACBD*::pPL2(*rmlACBD*)	DC367	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant complemented with pPL2(*rmlACBD*) (pDC313); Cm^r	This study
EGD-e Δ*rmlT*	DC492	EGD-e *rmlT* (*lmo1080*) deletion mutant	This study
EGD-e Δ*rmlT*::pPL2(*rmlT*)	DC553	EGD-e *rmlT* (*lmo1080*) deletion mutant complemented with pPL2(*rmlT*) (pDC550); Cm^r	This study
EGD-e Δ*tagO1*Δ*tagO2*::pLIV2(*tagO1*)		EGD-e *tagO1* (*lmo0959*) and *tagO2* (*lmo2519*) double deletion mutant complemented with pLIV2(*tagO1*), expressing *tagO1* under the control of an IPTG-inducible promoter; Cm^r	Eugster and Loessner 2012
EGD	BUG600	wild type; serotype 1/2a	Murray *et al.* 1926
EGD Δ*dltA*	BUG2182	EGD *dltA* (*LMON_0982*) deletion mutant	Mandin *et al.* 2005

I.9. Supplementary information

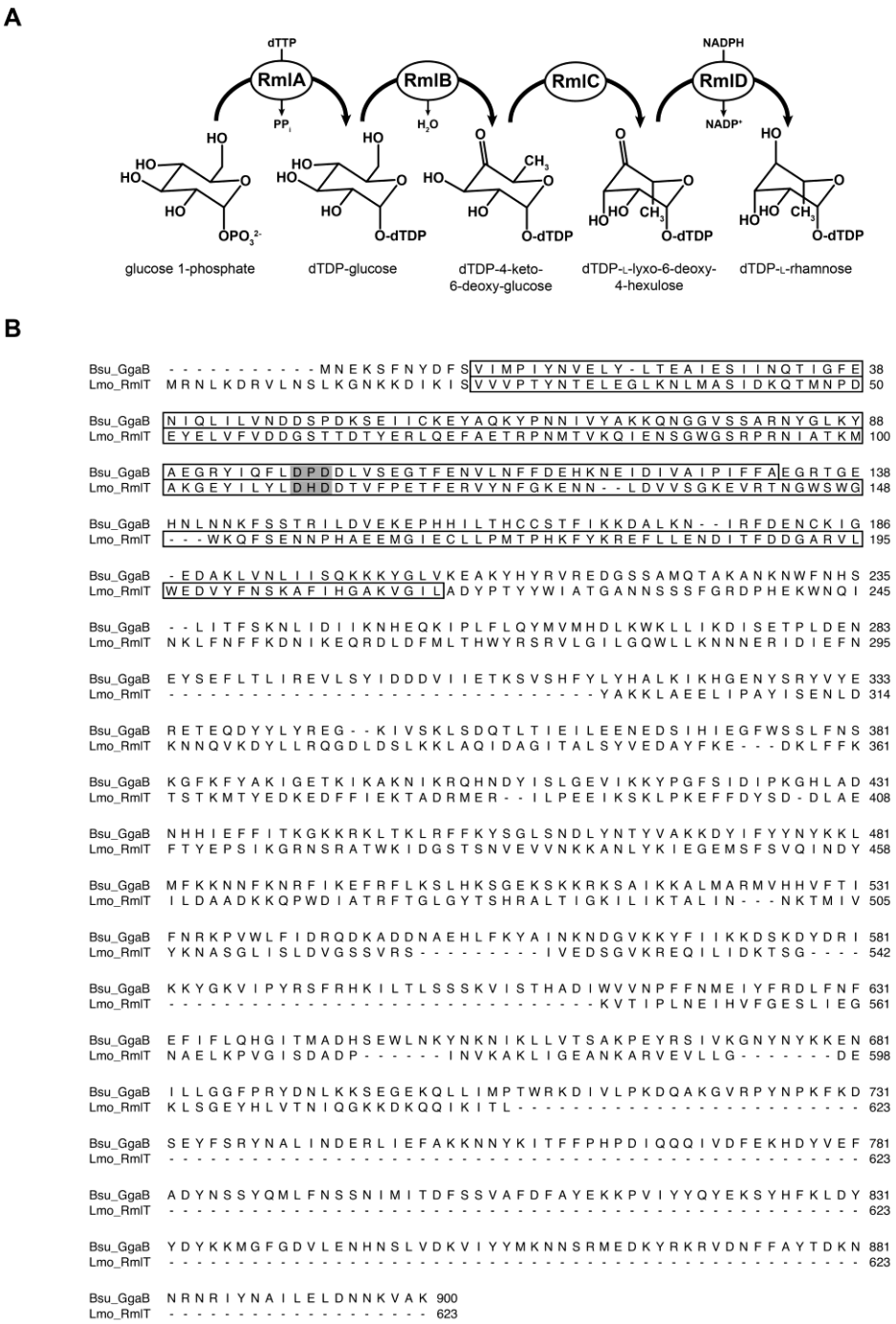

Fig. S1. Proteins involved in *Lm* WTA L-rhamnosylation. (**A**) Schematic diagram of the L-rhamnose biosynthesis pathway (adapted from (Giraud and Naismith 2000, Li *et al.* 2006)). Each of the RmlACBD proteins catalyzes one of the four reaction steps that convert glucose-1-phosphate into nucleotide-linked L-rhamnose. dTTP, thymidine triphosphate; PP_i, pyrophosphate; NADP, nicotinamide adenine dinucleotide phosphate. (**B**) Alignment of the amino acid sequences of *B. subtilis* 168 GgaB (GenBank: AAA73513.1) and *Lm* RmlT (GenBank: NP_464605.1). Boxed sequences correspond to the GT-A glycosyltransferase fold domain, as predicted by the NCBI Conserved Domain Search. The GT-A family signature DxD motif is highlighted in dark gray. The numbers indicate the position of the last amino acid in each line. Protein sequence alignments were obtained with ClustalW2 and edited with UCSF Chimera.

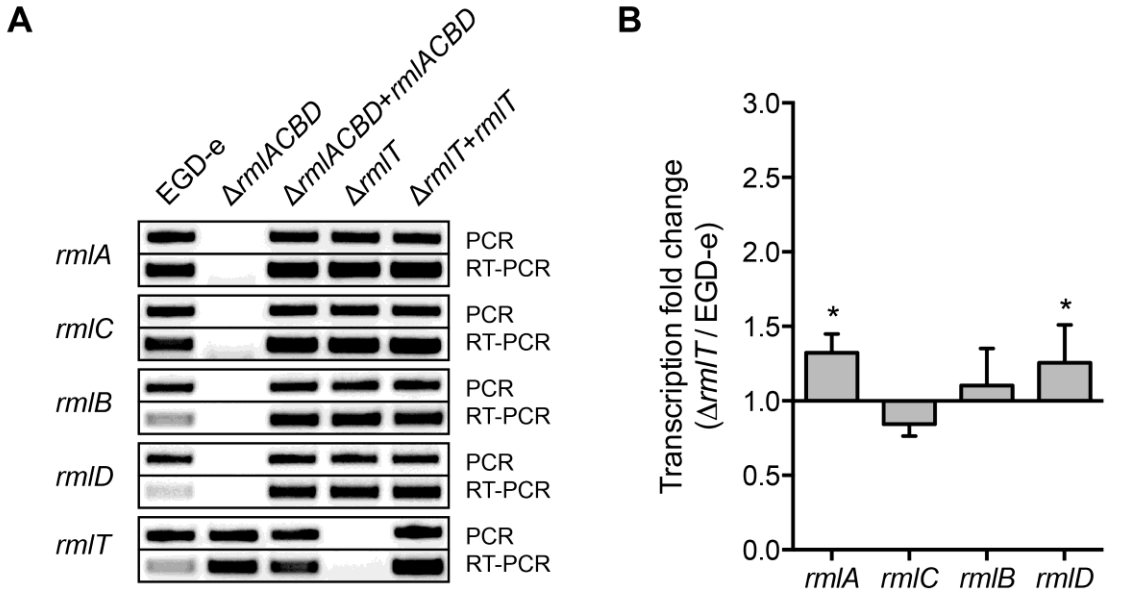

Fig. S2. Genetic characterization of *Lm* strains used in this study. (**A**) Genotypes and gene expression of the constructed *Lm* strains were confirmed by PCR and RT-PCR. (**B**) Comparison of the *rmlACBD* transcription levels in Δ*rmlT* versus wild type *Lm* strains by quantitative real-time PCR. Data represent the mean±SD of three independent analyses. *, $p \leq 0.05$.

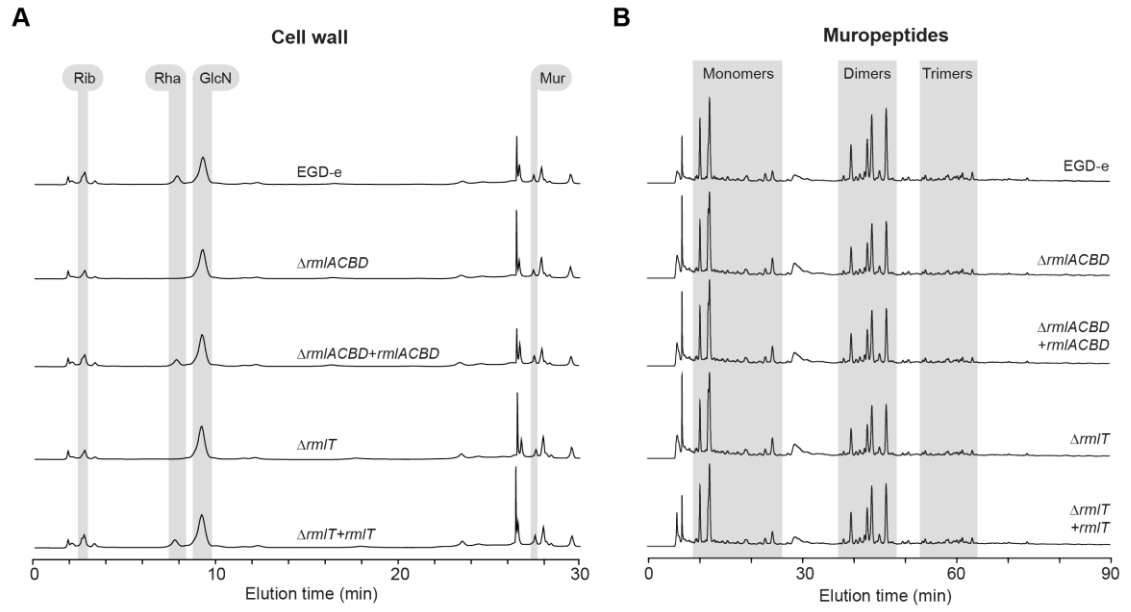

Fig. S3. HPLC analyses of the cell wall sugar and muropeptide composition from *Lm* strains. (**A**) HPAEC-PAD analysis of the sugar composition of cell wall purified from *Lm* strains. Samples were hydrolyzed in 3 M HCl (2 h, 95 ºC), diluted with water and lyophilized before injection into the HPLC equipment. Standards for ribitol (Rib), L-rhamnose (Rha), glucosamine (GlcN), and muramic acid (Mur) were eluted under identical conditions to allow peak identification. (**B**) Reverse-phase HPLC analysis of the muropeptide composition from different *Lm* strains, following overnight digestion of purified peptidoglycan samples with mutanolysin and reduction with $NaBH_4$. Muropeptide species (monomeric, dimeric, trimeric, etc.) were eluted with a 5–30% methanol gradient and detected by UV absorption at 206 nm.

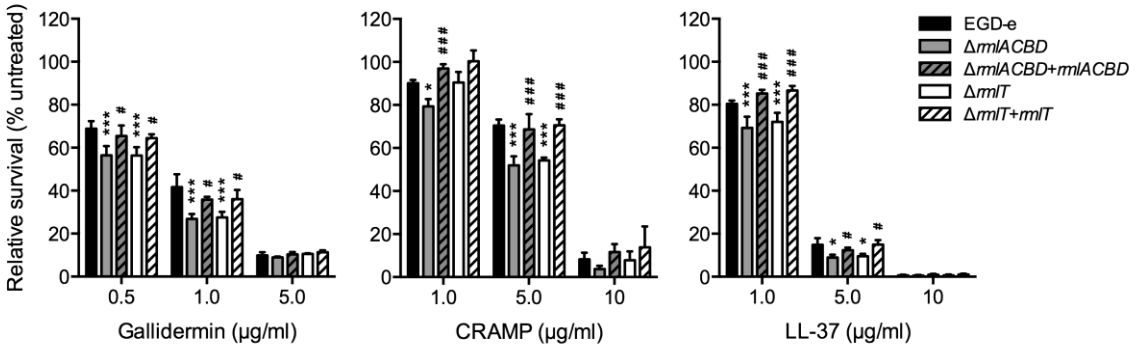

Fig. S4. Dose-dependent survival response of *Lm* strains to different AMPs. Quantification of viable bacteria after treatment of mid-exponential-phase *Lm* strains (2 h, 37 ºC) with increasing concentrations of gallidermin, CRAMP or LL-37. The average replicate values from AMP-treated samples were expressed as percentage of surviving bacteria relative to the values of the respective untreated control samples (set at 100). Data represent mean±SD of three independent experiments. Asterisks indicate statistical significance between wild type and mutant strains (*, $p \leq 0.05$; ***, $p \leq 0.001$), while hashes indicate statistical significance between mutant and respective complemented strains (#, $p \leq 0.05$; ###, $p \leq 0.001$).

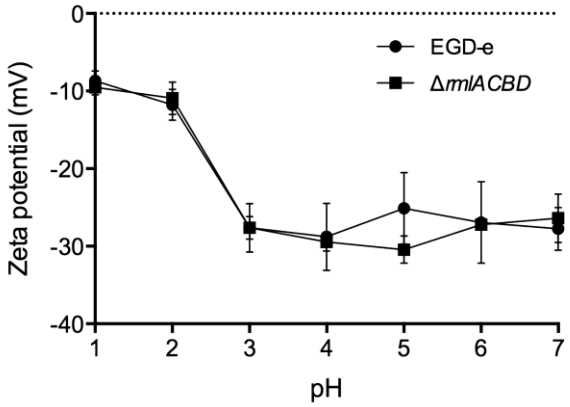

Fig. S5. Zeta potential profile of wild type and WTA L-rhamnosylation mutant *Lm* strains.

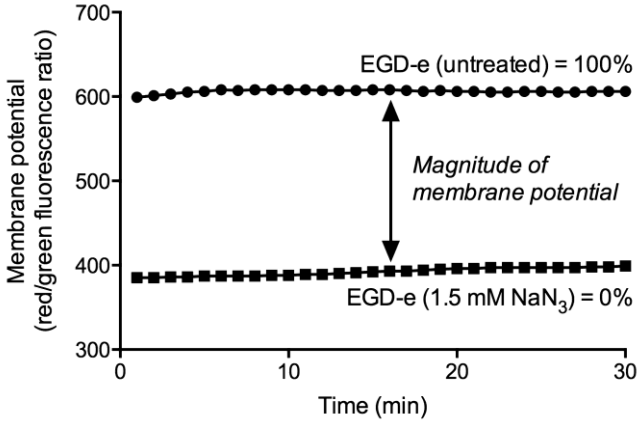

Fig. S6. Determination of the *Lm* membrane potential magnitude by flow cytometry. The membrane potential of untreated and sodium azide (1.5 mM)-treated suspensions of $DiOC_2(3)$-stained wild type EGD-e suspensions was analyzed (see Materials and Methods) to determine the red/green fluorescence ratio values corresponding, respectively, to a basal (100%) and null (0%) membrane potential.

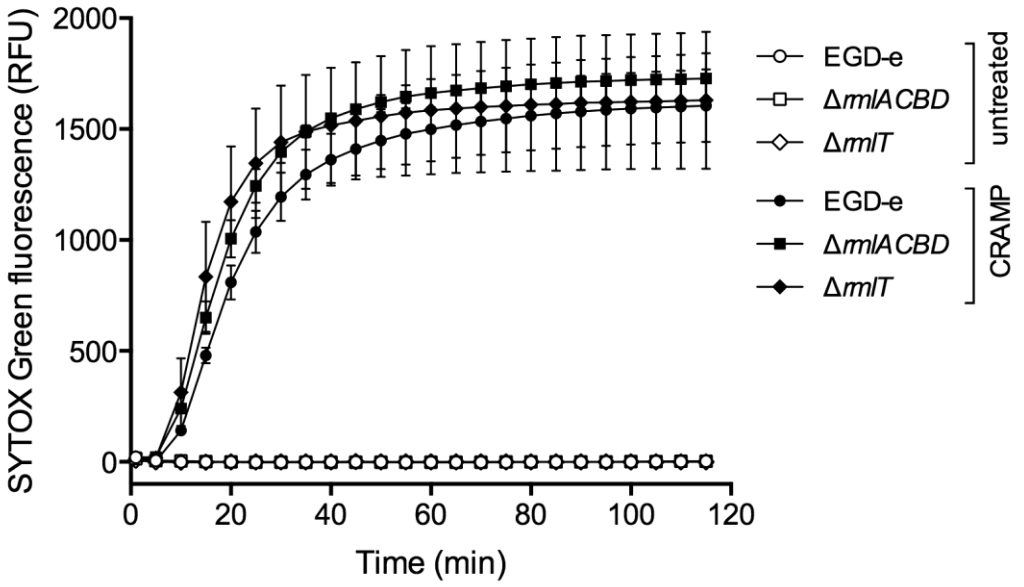

Fig. S7. SYTOX Green uptake kinetics of *Lm* strains in response to CRAMP-mediated membrane permeabilization. Exponential-phase bacteria were incubated (37 ºC) with PBS (white symbols) or 50 µg/ml CRAMP (black symbols), in the presence of 1 µM SYTOX Green, and the increase in green fluorescence emission was recorded over 115 min.

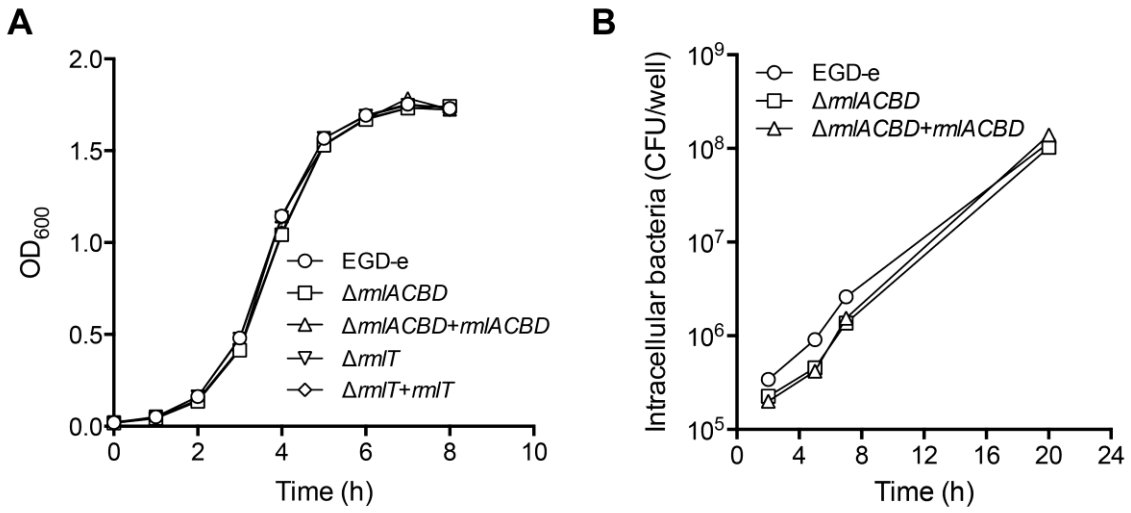

Fig. S8. Growth of *Lm* strains in broth and inside eukaryotic host cells. (**A**) Stationary-phase cultures were diluted 100-fold in BHI broth and incubated at 37 °C in aerobic and shaking conditions. Optical density values at 600 nm (OD_{600}) from each culture were measured every hour. (**B**) Intracellular multiplication in J774A.1 murine macrophages. Cells (2×10^5/well) were infected (45 min) with *Lm*, treated with 20 µg/ml gentamicin (75 min) and lysed at 2, 5, 7 and 20 h post-infection for quantification of intracellular viable bacteria in BHI agar.

Table S1. **Homology between the RmlACBD proteins of *Lm* EGD-e and other strains and species**[a]

Species[b] / Strain	Serovar	RmlA	RmlB	RmlC	RmlD
Lmo 10403S	1/2a	100	100	100	100
Lmo SLCC2755	1/2b	100	99	99.5	99.3
Lmo SLCC2372	1/2c	100	100	100	100
Lmo SLCC2479	3c	100	100	100	100
Lmo SLCC2482	7	100	98.7	99.5	99.3
Lse SLCC3954	1/2b	97.2	95.1	98.4	92.0
Smu UA159		74.6	45.7	28.6	51.6
Mtu H37Rv		58.3	46.7	33.5	34.3
Sen LT2		68.4	51.8	46.4	34.8
Sfl 2457T		70.8	51.5	48.0	35.9
Pae PAO1		69.1	52.4	47.2	32.2

[a] Values in percentage of amino acid identity as determined by protein-protein BLAST analysis.
[b] *Lmo*, *Listeria monocytogenes*; *Lse*, *Listeria seeligeri*; *Smu*, *Streptococcus mutans*; *Mtu*, *Mycobacterium tuberculosis*; *Sen*, *Salmonella enterica* serovar Typhimurium; *Sfl*, *Shigella flexneri*; *Pae*, *Pseudomonas aeruginosa*

Table S2. Primers

#	Name	Sequence (5' to 3')[a]
Construction of plasmids and screening of clones		
1	*rmlA*-A	TACGTCGACTGCTCAAATCGATGCTGG
2	*rmlA*-B	CGACGCGTCATTCTTTTCTCTCC
3	*rmlD*-C	ATACGCGTTTGGCAAGATGCTTTAGTTCG
4	*rmlD*-D	ATTAGATCTTAGTGGTCTCCACCAAGC
5	*rmlA*-F	GGCTACCACGTGAATGATCC
6	*rmlA*-R	AACTCACCACGTTCAGATGG
7	*rmlB*-F	GCAGCAGAATCTCATGTAGACC
8	*rmlB*-R	CCAGTTTCTCCAAGTGAACC
9	*rmlC*-F	ACATACGGTGAGTGGGAAGG
10	*rmlC*-R	AATCCGGATCATCGTAGGC
11	*rmlD*-F	TGGGAAGTAAACGTGGATGG
12	*rmlD*-R	CCAAACACCCATGAAGTACG
13	*rmlA*-G	ATACTATGCGGCCGCTTCATGTGTTTGGTGAAAGC
14	*rmlD*-H	GCGGTCGACACAATTATACGAATGCATCG
15	*rmlT*-A	ATAGTCGACCCTAAAGTTAATGGCAAAGCTCCTGC
16	*rmlT*-B	CGAATTCCATTATATCCTCCTAAAATAGATTAACAG
17	*rmlT*-C	CGAATTCTAAGAATGGAGAGAAAAGAATGAAAGG
18	*rmlT*-D	ACTAGATCTCAATTTCCATTAGTACGCCTCACTC
19	*rmlT*-F	TATTGCCACACGCTTTACCG
20	*rmlT*-R	CTTCCACGATTGAACGAACG
21	*rmlT*-G	TATCTGCAGGAGGGAAAACGTTAGGTAGC
22	*rmlT*-H	GCGGTCGACCTAGTTCCACTTCCTCCTGC
23	PL95	ACATAATCAGTCCAAAGTAGATGC
24	PL102	TATCAGACCTAACCCAAACCTTCC
Quantitative real-time PCR		
25	qPCR-*rmlA*-F	TTCTTGAAGCGTCTACCT
26	qPCR-*rmlA*-R	GCAGCCTCATCAATATACC
27	qPCR-*rmlB*-F	GTAGACCGTAGTATTATCAATCC
28	qPCR-*rmlB*-R	TCTCCAAGTGAACCATACA
29	qPCR-*rmlC*-F	TATTCAAGATAACCACTC
30	qPCR-*rmlC*-R	TCAACAACTACATCATAA
31	qPCR-*rmlD*-F	AGATTCTGTAGATATTGTGGAT
32	qPCR-*rmlD*-R	CATCTTCTGCTGCTTCTA
32	qPCR-16S-F	GCGTAGATATGTGGAGGAAC
33	qPCR-16S-R	CAGGCGGAGTGCTTAATG

[a] Restriction sites underlined

PART II

L-Rhamnosylation of *Listeria monocytogenes* wall teichoic acids is required for efficient surface anchoring of GW proteins

II.1. Introduction

In Gram-positive bacteria, a large portion of surface proteins associate non-covalently with cell envelope components through interactions that are commonly mediated by protein domains containing repeated sequences. Interestingly, many of these proteins appear to be associated with autolytic functions (Scott and Barnett 2006, Bierne and Cossart 2007), indicating that this type of labile, reversible cell surface association provides some sort of positional flexibility for bacterial cell wall-degrading enzymes that is key for their optimal activity. Repeat domains like the LysM domain anchor proteins directly to the peptidoglycan (Buist *et al.* 2008), while others have affinity for secondary cell wall polymers, such as TAs. For instance, the pneumococcal virulence-promoting PspA adhesin and LytA amidase have similar C-terminal choline-binding repeats, which are necessary and sufficient for their attachment to the *S. pneumoniae* choline-decorated LTAs (Holtje and Tomasz 1975, Yother and White 1994). Similarly, proteins carrying GW repeat domains were shown or at least strongly suggested to interact with LTAs, as observed in the cases of the *S. aureus* autolysin Atl (Yamada *et al.* 1996), and the *Lm* invasin InlB and autolysin Ami (Jonquières *et al.* 1999).

WTAs also dictate the localization and control the activity of autolytic proteins at the bacterial surface (Brown *et al.* 2013). Characterization of *S. aureus* WTA mutants revealed anomalies in autolysis levels and in the ability to properly form septa and/or complete cell division (Vergara-Irigaray *et al.* 2008, Schlag *et al.* 2010, Biswas *et al.* 2012, Qamar and Golemi-Kotra 2012). Moreover, the particular contribution of WTA substituents to *S. aureus* autolysis was contrasting: whereas D-alanylation is essential for proper autolytic activity (Peschel *et al.* 2000), the impairment of WTA glycosylation with Glc*N*Ac did not perturb this process (Brown *et al.* 2012), indicating that sugar substituents are not involved in WTA-mediated regulation of autolysis. In *Lm*, LTA D-alanylation is required for cell adhesion and virulence *in vivo* (Abachin *et al.* 2002), however its role in autolysis was never addressed. Likewise, information regarding the contribution of *Lm* WTAs – and of their glycosidic substituents – to this process is currently nonexistent. Therefore, we decided to study the involvement of this particular WTA tailoring mechanism in the spatial and functional regulation of *Lm* autolysis.

We showed that an *Lm* mutant strain lacking L-rhamnosylated WTAs (Δ*rmlACBD*) displays a reduced autolysis rate in comparison with a wild type strain. This phenotype appears to be linked to a prominent decrease of the *Lm* cell surface-associated levels of the autolysin Ami. Moreover, we observed that this decrease is concurrent with secretion of Ami to culture supernatants, suggesting that WTA L-rhamnosylation is necessary for efficient association of Ami to the *Lm* cell surface. To determine if other *Lm* GW proteins were similarly affected, we screened for WTA L-rhamnosylation-dependent variations in the surface association of the remaining eight GW repeat-containing proteins encoded in the *Lm* genome (Cabanes *et al.* 2002). Besides Ami, only InlB showed an anchoring mechanism dependent on the *Lm* WTA L-rhamnosylation status, albeit to a lesser degree. This shift in the relative distribution of this major *Lm* invasin may be responsible for the impaired entry of the Δ*rmlACBD* strain in different epithelial cell lines. These data reveal novel roles for WTA L-rhamnosylation in *Lm* biology, such as supporting autolytic processes and promoting host cell invasion, via its contribution to the efficient anchoring of a particular group of surface proteins.

II.2. Results

II.2.1. WTA L-rhamnosylation-deficient *Lm* is less autolytic due to deficient surface anchoring of the autolysin Ami

To assess the contribution of WTA L-rhamnosylation to autolytic processes in *Lm*, we performed an *in vitro* autolysis assay through which we compared the lysis kinetics of wild type and WTA L-rhamnosylation-deficient (Δ*rmlACBD*) *Lm* strains shaken at 37 ºC in a neutral pH buffer. We observed that bacterial suspensions containing the Δ*rmlACBD* strain clarified at a slower pace than the ones containing the wild type strain (Fig. 25A), indicating that autolysis was decreased in *Lm* populations lacking L-rhamnosylated WTAs. This WTA L-rhamnosylation dependence for normal autolytic activity was corroborated by the lysis profile of the complemented Δ*rmlACBD* strain (Δ*rmlACBD*+*rmlACBD*) in the same conditions, which showed reversion (albeit partial) of the mutant phenotype towards a wild type-like autolytic phenotype (Fig. 25A).

The reduced autolytic levels observed in WTA L-rhamnosylation mutant bacteria could be the result of a decrease in the surface localization and/or activity of autolytic enzymes. To determine which of these hypotheses was true we analyzed the surface proteomes – in particular, non-covalently cell wall-attached proteins, which include most autolysins – of wild type and Δ*rmlACBD* bacteria, in search of significant protein content changes between both strains. After SDS-mediated retrieval of surface proteins from mid-exponential-phase bacteria, extracts were resolved by SDS-PAGE and proteins visualized by Coomassie staining. We observed a striking reduction in the amount of an abundant 75–100 kDa protein in the Δ*rmlACBD* sample relative to the same band present in the wild type extract (Fig. 25B). Moreover, this decrease is dependent on the presence of L-rhamnosylated WTAs, since the amount of this protein in Δ*rmlACBD*+*rmlACBD* bacteria increased to levels similar to those observed in wild type extracts (Fig. 25B). Through peptide mass fingerprinting, this protein was identified (confidence index of 100%) as the *Lm* virulence-associated autolytic amidase Ami (McLaughlan and Foster 1998, Milohanic *et al.* 2001). This decrease in the amount of surface-associated Ami in Δ*rmlACBD* bacteria agrees with the lower levels of

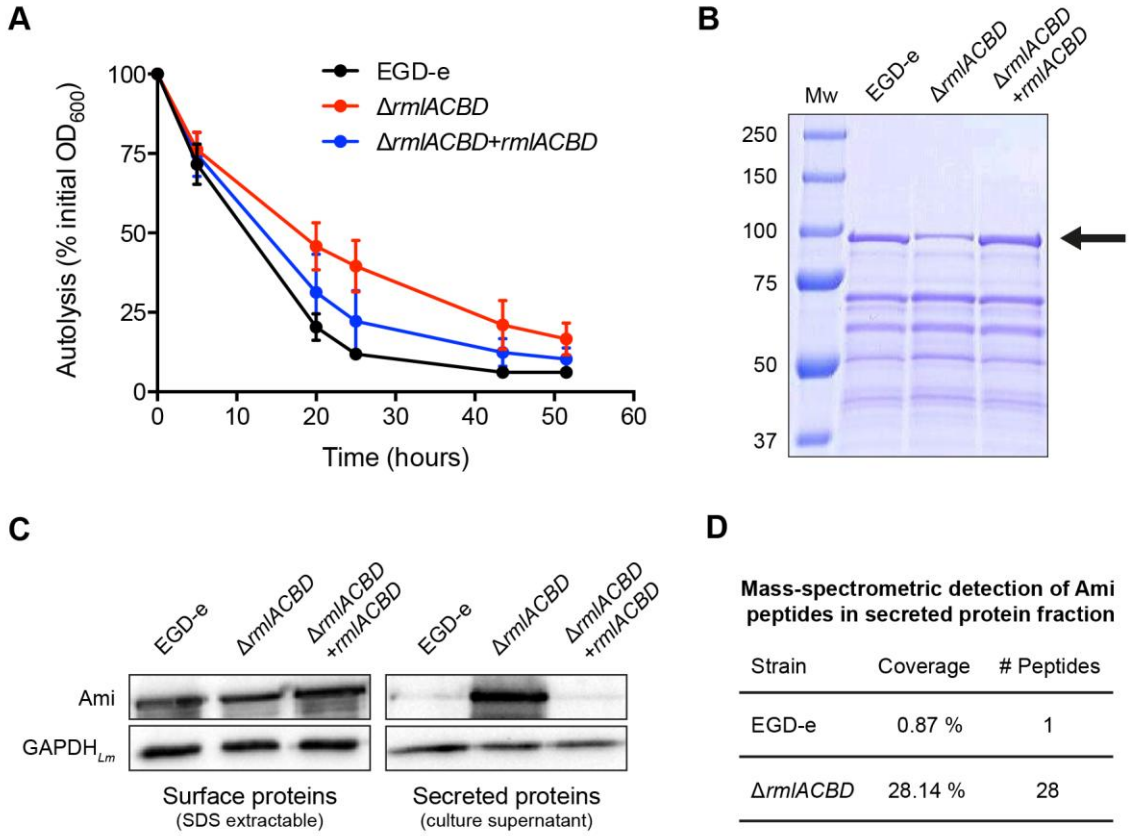

Fig. 25. WTA L-rhamnosylation-deficient *Lm* is less autolytic due to deficient surface anchoring of the autolysin Ami. (A) *Lm* strains grown to the exponential phase (OD_{600} 1.0) were washed and resuspended in 1 volume of 50 mM glycine buffer (pH 8.0). Bacterial suspensions were incubated at 37 ºC with shaking and autolysis was measured at different time points as the decrease in OD_{600} relative to the initial value, set as 100%. **(B)** Non-covalently associated surface proteins were extracted from mid-exponential-phase *Lm* strains with 2% SDS (37 ºC, 30 min), concentrated, resolved by SDS-PAGE and stained with Coomassie Brilliant blue. Black arrow indicates a protein with strain-dependent quantity changes that was identified by peptide mass fingerprinting as Ami. Mw, molecular weight ladder (standard band weight indicated in kDa). **(C)** Non-covalently associated surface proteins (obtained as in B) and secreted proteins (recovered from culture supernatant) from *Lm* strains were analyzed by Western blot to detect the levels of Ami. The listerial GAPDH ($GAPDH_{Lm}$) protein was used as loading control. Blots are representative of two independent experiments. **(D)** Ami is highly secreted to the surrounding environment in the absence of L-rhamnosylated WTAs. Data from a comparative secretomic analysis of wild type (EGD-e) and Δ*rmlACBD* strains shows an increase in the number of Ami-derived peptides detected in the Δ*rmlACBD* culture supernatant sample.

autolysis observed in this strain, indicating that WTA L-rhamnosylation is required to maintain a certain amount of Ami at the *Lm* cell surface and thus support a physiological level of autolytic activity.

Taking into account that Ami is a member of the GW protein family in *Lm* (Cabanes *et al.* 2002) and that TAs are important surface anchors of proteins bearing this class of cell wall-binding repeat domains (Yamada *et al.* 1996, Jonquières *et al.* 1999), we investigated if the diminished levels of Ami at the cell surface of WTA L-rhamnosylation mutant bacteria were a consequence of a defect

in the cell wall association of this protein. To do this, we performed a Western blot analysis of the (SDS-extractable) surface protein and the secreted protein fractions from both wild type and Δ*rmlACBD* strains, using an anti-Ami antiserum to detect this protein. Immunoblot results confirmed the SDS-PAGE results (Fig. 25B), showing the same drop of surface-associated Ami levels in mutant bacteria as compared to wild type bacteria (Fig. 25C). Conversely, a significantly higher amount of Ami was detected in the secreted protein fraction of the Δ*rmlACBD* strain (Fig. 25C). Mutant complementation restored the protein levels in each fraction to those observed in samples obtained from wild type bacteria (Fig. 25C). Further validation of these results was achieved with a comparative analysis of the secretomes of wild type and Δ*rmlACBD* strains, performed in collaboration with the group of Francisco García-del Portillo (CNB-CSIC, Madrid), which revealed a significant increase in the amount of Ami-derived peptides detected in Δ*rmlACBD* samples, relative to those in wild type samples (Fig. 25D).

Altogether, these results definitively confirm that, in the absence of WTA L-rhamnosylation, Ami protein molecules are not properly affixed to the *Lm* cell surface and, as a result, end up being secreted into the surrounding environment. This supports the hypothesis that the decoration of *Lm* WTAs with L-rhamnose controls the surface-associated levels of Ami, and concomitantly Ami-dependent autolytic events, by promoting their efficient attachment to the cell envelope.

II.2.2. Study of the WTA L-rhamnosylation-dependent surface localization of *Lm* GW proteins

To determine if WTA L-rhamnosylation is also important for the correct surface anchoring of other *Lm* GW proteins, we screened the surface-associated and secreted protein fractions from wild type and Δ*rmlACBD* strains expressing tagged variants of all nine GW proteins encoded in the *Lm* genome (Cabanes *et al.* 2002) in order to detect strain-dependent differences in the partition of each protein. To do this, we first generated a plasmid construct based on the listerial site-specific integrative pPL2 (Lauer *et al.* 2002), which enabled the expression of N-terminally FLAG-tagged sequences under the control of the native *ami* promoter (Fig. 26A). Nine plasmid constructs were derived from this one by cloning

individually the GW repeat domain-encoding sequence of each GW protein – Ami (Lmo2558), Auto (Lmo1076), InlB (Lmo0434), Lmo1215, Lmo1216, Lmo1521, Lmo2203, Lmo2591 and Lmo2713 – downstream the FLAG tag sequence. Finally, each plasmid was introduced and stably integrated into the chromosome of wild type and Δ*rmlACBD* bacteria, giving rise to 18 new strains.

Following the fractionation of mid-exponential-phase cultures to obtain extracts of secreted and (non-covalently) surface-associated proteins, samples were analyzed by Western blot to detect FLAG-tagged proteins. Using an anti-FLAG probe, we were able to visualize bands with the correct molecular weights from every strain (Fig. 26B), indicating that all plasmid constructs were functional and that bacteria were successfully expressing the tagged GW protein truncates. When comparing protein distribution between strains in each fraction, we observed different situations. Concerning the GW domain of Ami (Ami_{GW}), the largest of all *Lm* GW proteins, with eight repeats, we confirmed the previous observations that showed a sharp reduction of surface-associated protein levels and a concomitant rise in Ami secretion in Δ*rmlACBD* bacteria (Fig. 26B). A similar outcome (i.e. lower surface association/increased secretion) was verified with $InlB_{GW}$, although the difference was not as large as with Ami_{GW}. As previously reported (Lingnau *et al.* 1995, Jonquières *et al.* 1999), FLAG-tagged $InlB_{GW}$ was also found in both fractions, showing the dual character of InlB. In contrast, the similarly sized $Auto_{GW}$ and $Lmo2591_{GW}$ showed no variation in their respective protein levels between strains. However, while the former protein was exclusively detected in the surface-associated fraction, the latter was only present in the secreted protein extracts (Fig. 26B). The remaining (and smallest) GW protein truncates ($Lmo1215_{GW}$, $Lmo1216_{GW}$, $Lmo1521_{GW}$, $Lmo2203_{GW}$ and $Lmo2713_{GW}$), were all detected solely in the secreted fractions of both strains, like $Lmo2591_{GW}$ (Fig. 26B).

These results indicate that other *Lm* GW proteins besides Ami rely on WTA L-rhamnosylation for proper attachment to the bacterial surface. Moreover, they suggest that whereas proteins with large-sized GW repeat domains display a WTA L-rhamnosylation-dependent mechanism of *Lm* cell surface association, this dependence seems to be lost in proteins with increasingly smaller GW domains, as such proteins are frequently found to be fully secreted.

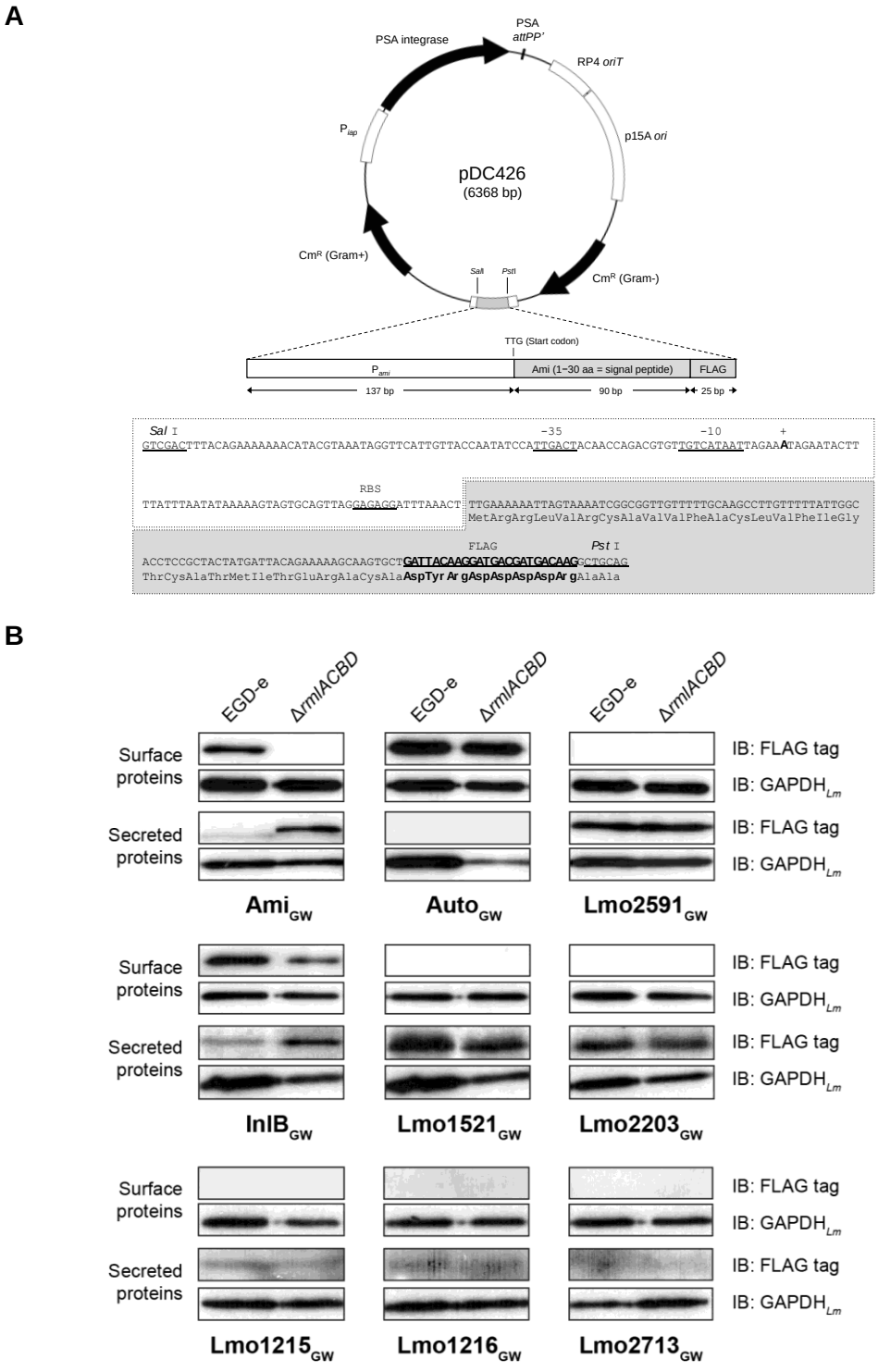

Fig. 26. Study of the WTA L-rhamnosylation-dependent surface localization of *Lm* GW proteins. (A) Map of the plasmid template used to enable *ami* promoter (P_{ami}, white boxed sequence)-dependent expression of N-terminally FLAG-tagged proteins targeted for secretion by the Ami signal peptide (gray boxed sequence). The vector backbone is derived from pPL2 plasmid (Lauer *et al.* 2002). Other sequence elements, such as the -35 and -10 promoter boxes, the transcription start site (+), the ribosome-binding site (RBS), and unique restriction enzyme sites are underlined. **(B)** Mid-exponential-phase cultures of wild type and Δ*rmlACBD* bacteria expressing FLAG-tagged proteins corresponding to the GW repeat domains of each of the nine *Lm* GW proteins were processed for the recovery of secreted proteins and non-covalently associated surface proteins. Proteins extracts were concentrated and analyzed by Western blot, using an anti-FLAG tag antibody to detect GW domain proteins and compare their levels between strains and fractions. The listerial GAPDH protein was used as loading control. IB, immunoblotting antibody.

II.2.3. WTA L-rhamnosylation is required for host cell invasion

The previous results showed that only two members of the *Lm* family of GW proteins were differentially anchored to the bacterial surface in a WTA L-rhamnosylation-dependent fashion: the autolysin Ami and the host cell invasion-promoting protein InlB. We proposed a link between the reduced amount of surface-associated Ami and the lower levels of autolysis observed in Δ*rmlACBD* bacteria, so we sought to investigate the effect of the decreased bacteria-associated InlB levels in the ability of *Lm* to interact with and enter into host cells.

Having confirmed the increased secretion of the native, full-length InlB protein in Δ*rmlACBD* bacteria by Western blot, using an InlB-specific antibody (Fig. 27A), we began this study by testing the cell-adhesive potential of *Lm* lacking

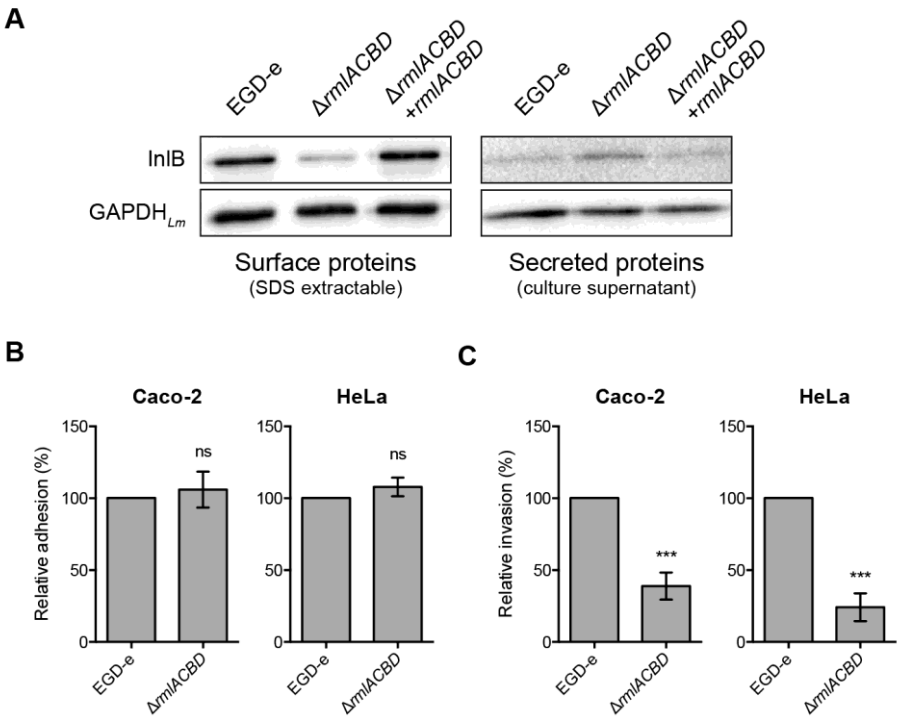

Fig. 27. WTA L-rhamnosylation is required for host cell invasion but not adhesion. (A) Non-covalently associated surface and secreted protein fractions from *Lm* strains were analyzed by Western blot to detect the InlB. The listerial GAPDH (GAPDH$_{Lm}$) protein was used as loading control. Blots are representative of two independent experiments. **(B, C)** Caco-2 and HeLa cell monolayers were used to evaluate the host cell adhesion and invasion capacity of wild type (EGD-e) and WTA L-rhamnosylation-deficient (Δ*rmlACBD*) *Lm* strains. For adhesion assays (B), cells were infected (MOI 50) for 30 min (37 ºC, 7% CO_2), washed thoroughly and lysed in cold 0.2% Triton X-100 for CFU quantification of cell-associated bacteria. For invasion assays (C), cells were infected (MOI 50) for 1 h (37 ºC, 7% CO_2), treated with 20 µg/ml gentamicin for 1.5 h (37 ºC, 7% CO_2), washed thoroughly and lysed in cold 0.2% Triton X-100 for CFU quantification of intracellular bacteria. Data are represent as mean±SD (n=3) and presented as percentage relative to the wild type value, set at 100. Statistical analyses were performed using an unpaired, two-tailed *t*-test (ns, not significant; ***, p<0,001).

L-rhamnose-decorated WTAs. For this, human epithelial cell lines (Caco-2 and HeLa) were briefly incubated with either wild type or Δ*rmlACBD Lm* strains and after several washes the number of cell-associated bacteria was quantified. Mutant bacteria evidenced a cell-binding ability comparable to that of their wild type congeners, indicating that the surface depletion of Ami and InlB does not interfere with the attachment efficiency of WTA L-rhamnosylation-deprived *Lm* (Fig. 27B). Next, we compared the invasiveness of these strains using the same eukaryotic cell lines. Unlike their wild type-like adhesive properties, Δ*rmlACBD* bacteria displayed a significant reduction of their intracellular levels in both Caco-2 and HeLa cells (<40% of wild type) after 2.5 hours of infection (Fig. 27C), revealing a strong impairment of *Lm*-induced uptake by target cells in the absence of L-rhamnosylated WTAs. This attenuated phenotype correlates with the decrease in *Lm* surface levels of InlB observed in the Δ*rmlACBD* strain, as *Lm* Δ*inlB* mutants were previously shown to be weakly to non-invasive in these cell lines (Dramsi *et al.* 1995, Ireton *et al.* 1996).

Importantly, these results unveil a novel link between WTA L-rhamnosylation and *Lm* virulence via its contribution towards the maintenance of optimal levels of the surface-associated invasin InlB.

II.3. Discussion

The Gram-positive cell envelope is host to a vast array of proteins whose diversity of structural and functional roles is extremely important not only for cell surface maintenance and metabolism but also for overall bacterial physiology and viability (Navarre and Schneewind 1999). Several proteins interact with the bacterial surface in a non-covalent manner that is often based in the affinity between protein repeat-containing domains and specific cell envelope components (Desvaux *et al.* 2006). Interestingly, a considerable number of proteins using this mechanism of surface association is known or predicted to have autolytic (i.e. cell wall-degrading) functions (Desvaux *et al.* 2006, Scott and Barnett 2006, Bierne and Cossart 2007). TAs have been either identified or presumed to perform cell envelope protein-anchoring functions in different species (Holtje and Tomasz 1975, Yother and White 1994, Yamada *et al.* 1996, Jonquières *et al.* 1999). In some cases, this role was found to be determined by TA substituents, such as choline in streptococci (Holtje and Tomasz 1975) and D-alanine in *S. aureus* (Peschel *et al.* 2000).

In the case of *Lm*, there are no evidences regarding TA-mediated anchoring of autolysins, although it has been suggested (Asano *et al.* 2012) that the virulence-associated amidase Ami (Milohanic *et al.* 2001) may bind to LTAs via its GW repeat domain, in a process similar to that of InlB (Jonquières *et al.* 1999). The lack of information concerning the particular involvement of *Lm* WTAs and their substituents in the surface positioning and activity of autolysins prompted us to address this question. This work confirmed the existence of a link between *Lm* WTA L-rhamnosylation and autolytic activity. It specifically showed that this tailoring mechanism is required to support basal levels of autolysis through the efficient attachment of Ami to the *Lm* cell surface. In addition, it revealed that InlB, a major cell invasion-promoting factor that contains a GW repeat domain similar to Ami, is also less associated with the surface of *Lm* lacking L-rhamnosylated WTAs. Importantly, this finding is consistent with a significant impairment of host cell invasion levels observed in these mutant bacteria.

To answer the question of whether L-rhamnosylation of *Lm* WTAs contributes to the mechanisms of bacterial self-degradation, a simple *in vitro* autolysis assay

revealed that the rate of self-induced lysis was diminished in bacteria devoid of this WTA glycosylation mechanism, and that its reintroduction was sufficient to restore normal levels of autolysis. A similar observation was made with *S. aureus dltA* mutants, unable to perform D-alanylation of TAs (Peschel *et al.* 2000). In this case, it was suggested that the strongly anionic D-alanine ester-free TAs bind avidly the positively charged autolysins (Fischer *et al.* 1981), inhibiting their action through entrapment. However, a parallel study using a *dltC* mutant in another *S. aureus* strain background and slightly different experimental conditions provided an opposite phenotype, i.e. enhanced cell lysis (Nakao *et al.* 2000). This was also reported in D-alanylation mutant strains of *L. lactis* and *L. plantarum* (Steen *et al.* 2005, Palumbo *et al.* 2006). In both situations, unrestrained activity of a major autolysin is pointed as the reason for the mutant phenotypes. In the *L. lactis* mutant, this was attributed in part to a reduced HtrA-mediated degradation of the AcmA autolysin (Steen *et al.* 2005), while the LTA polymers of the *L. plantarum dltA* mutant were longer and heavily glucosylated (Palumbo *et al.* 2006). The conflicting behaviors observed in different bacterial species deficient in the same mechanism highlight the complexity of the mechanisms linking TAs and autolysin activity.

Reduced surface levels of autolytic proteins can explain the decline in the levels of autolysis observed in WTA L-rhamnosylation-deprived *Lm*. Our data from an SDS-PAGE analysis of non-covalently associated surface protein extracts from WTA L-rhamnosylation mutant *Lm* revealed a striking drop in the amount of a high-molecular weight protein (~100 kDa) in comparison with its levels in wild type bacterial extracts. We identified this protein as Ami, a 99-kDa autolytic *N*-acetylmuramoyl-L-alanine amidase (McLaughlan and Foster 1998), whose immature precursor weighs 102 kDa before signal peptide cleavage. Ami is the biggest of nine *Lm* surface proteins containing a domain with GW module repeats (Cabanes *et al.* 2002). Its considerable length (917-aa precursor) is conferred by an extensive C-terminal domain with eight (or four pairs of) GW module repeats (McLaughlan and Foster 1998, Milohanic *et al.* 2001, Cabanes *et al.* 2002). InlB, another *Lm* GW protein, is reversibly attached to the cell envelope via interaction with LTAs (Jonquières *et al.* 1999). As mentioned before, it is suggested that Ami also associates with the *Lm* cell surface by binding to LTAs (Braun *et al.* 1997,

Jonquières *et al.* 1999, Asano *et al.* 2012), although this still requires direct experimental confirmation.

Considering the reduction of the surface levels of Ami in *Lm* cells lacking L-rhamnosylated WTAs and previous studies supporting the participation of TAs in the non-covalent anchoring of surface proteins bearing repeat domains, we investigated the potential involvement of WTAs – and in particular the contribution of their decoration with L-rhamnose – in the interaction of Ami with the *Lm* surface. Our data from immunoblot analysis of the *Lm* surface protein and secreted protein fractions, complemented with a high-throughput secretomic study of the wild type and mutant strains, demonstrated that the absence of WTA L-rhamnosylation results in significant oversecretion of Ami, which explains the fate of the missing surface-associated protein. Although it would be interesting to verify if there are changes in the total levels of Ami expression in both strains, it seems clear that there is a shift in the spatial distribution of Ami, from a predominant *Lm* surface localization to a chiefly secreted form. This finding uncovers a rather preeminent role for WTA L-rhamnosylation in the mechanisms of surface protein anchoring, since to our knowledge there was no prior evidence of WTA glycosidic substituents having such a significant influence, either direct or indirect, on surface protein binding levels. In this context, it would be important to confirm the role of LTAs as prime surface anchors for Ami and investigate if L-rhamnosylated WTAs can act as some sort of secondary structures that help stabilize the Ami-LTA interaction. For the latter hypothesis, surface-associated Ami protein levels could be quantified in an *Lm* WTA mutant strain, although the severe growth and morphological defects characteristic of this strain should be taken into account (Eugster and Loessner 2012). Interestingly, one report characterizing Ami orthologues produced by *Lm* strains of serotypes 1/2a and 4b showed that (i) each protein only bound efficiently to the surface of bacteria from its own serotype, and that (ii) the GW domain sequences are homologous within serotypes with similar WTA structures (Milohanic *et al.* 2004). This highlights a clear role for WTAs and its sugar substituents in the anchoring of Ami and potentially of other GW proteins.

Besides Ami and InlB, *Lm* encodes seven other GW proteins (Cabanes *et al.* 2002). Among these, only one, Auto (Lmo1076), has been characterized as an autolysin (Cabanes *et al.* 2004) that, unlike Ami, possesses NAGase activity

(Bublitz *et al.* 2009). We attempted to evaluate a potential WTA L-rhamnosylation dependence in the cell surface anchoring mechanism of *Lm* GW proteins other than Ami. In our approach, each protein was represented solely by its GW repeat domain for two reasons: (i) to assess its specific contribution in full-length protein binding, and (ii) to prevent toxicity/lethality during the cloning stages, as the catalytic domains of all but two GW proteins (InlB and Lmo2713) are predicted to have bacteriolytic activities (Bierne and Cossart 2007). Indeed, previous studies were troubled by unsuccessful attempts to clone either the full-length or the N-terminal fragments of Ami (Braun *et al.* 1997, McLaughlan and Foster 1998). Recently, Asano and colleagues were successful in cloning and expressing recombinant Ami forms containing the amidase domain (Asano *et al.* 2012), showing that it is possible to express heterologously the full-length form of this autolysin.

Our results show that the effect observed with native Ami was reproduced with only its GW domain (Ami_{GW}), indicating that this repeat-rich region is entirely responsible for *Lm* surface anchoring of full-length Ami and strengthening the supportive role of L-rhamnosylated WTAs in this process. In addition, they revealed that, besides Ami_{GW}, only $InlB_{GW}$ displayed increased secretion in the absence of WTA L-rhamnosylation. This effect was also observed with the native full-length InlB protein. However, the extent of this oversecretion is not as big as the one observed with Ami_{GW}, which is not surprising considering that InlB is already found in both bacterium-associated and secreted forms in wild type conditions (Braun *et al.* 1997, Jonquières *et al.* 1999). While none of the remaining seven GW domains showed strain-dependent variations of their protein levels, they were exclusively detected either in the *Lm* surface ($Auto_{GW}$) or in the secreted protein fraction (all others). It is interesting to observe GW domains of different proteins (Auto and Lmo2591), but with similar molecular weights and equal number of repeats, displaying totally opposite localizations. This strongly suggests that other sequence elements besides the number of GW module repeats (Braun *et al.* 1997) may also determine the spatial localization of these proteins.

Immunoblot detection of the FLAG-tagged GW domains became increasingly difficult with decreasing molecular weight, indicating that smaller proteins are not as well expressed as the larger ones or that they are not as easily recovered from

culture supernatants during precipitation. Since all GW domains were expressed under the control of the same promoter (*ami*), the second hypothesis seems more plausible. Although this analysis requires further confirmation, its results provide significant insights into the anchoring mechanisms of each *Lm* GW protein.

As a major determinant of *Lm* internalization into different types of host cells (Dramsi *et al.* 1995, Lingnau *et al.* 1995, Ireton *et al.* 1996, Parida *et al.* 1998), we further investigated the potential effects of enhanced InlB secretion regarding this process. Our data demonstrated that a WTA L-rhamnosylation *Lm* mutant strain is not affected in its ability to adhere to epithelial cell monolayers but is significantly attenuated in its self-induced cellular uptake levels. Functional characterization of Ami demonstrated its contribution towards *Lm* adhesion to target cells (Milohanic *et al.* 2001). However, this role appears to be secondary as it only becomes relevant in a Δ*inlAB* background, and varies in a cell type-dependent manner (Milohanic *et al.* 2001). In this context, we could expect decreased host cell adhesion levels in *Lm* WTA L-rhamnosylation mutants, since Ami levels are critically reduced at their surface and InlB is also partially depleted. However, it is likely that the remaining amount of anchored InlB, together with InlA, are sufficient to maintain optimal levels of eukaryotic cell association. Although InlB occurs normally in both surface-attached and secreted forms, the bacterium-bound form is more preponderant at triggering host cell internalization of *Lm* (Braun *et al.* 1998), which could explain the attenuated phenotype of the WTA L-rhamnosylation mutant strain. Moreover, InlB is known to interact with other eukaryotic cell surface components to further promote bacterial invasion (Braun *et al.* 2000, Jonquières *et al.* 2001). In the case of GAGs, this interaction takes place through the GW domain (Jonquières *et al.* 2001). We can speculate that excessive amounts of soluble InlB close to the site of *Lm* association with the host cell surface may saturate these InlB-binding eukaryotic partners to a point where it hinders *Lm* internalization.

Caco-2 and HeLa cell lines have been extensively used for the study of *Lm* internalization mechanisms primarily dependent on the engagement of either InlA (Caco-2) or InlB (HeLa). Although Caco-2 cells also express the InlB receptor c-Met (Pizarro-Cerdá *et al.* 2012), our results showed a cell type-independent invasion defect, suggesting that the bacterial aspect of the InlA-mediated

internalization pathway may also be affected in the absence of WTA L-rhamnosylation.

In conclusion, this work has demonstrated the contribution of WTA L-rhamnosylation to important physiological and virulence processes (invasion of host cells) in *Lm*, through a newly identified role in the anchoring and stabilization of non-covalently bound surface proteins sharing a common cell surface-binding repeat domain.

II.4. Materials and methods

II.4.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *Lm* and *E. coli* strains were routinely cultured aerobically at 37 ºC in brain heart infusion (BHI, Difco) and lysogeny broth (LB) media, respectively, with shaking. When appropriate, the following antibiotics were added as selective agents: ampicilin (Amp), 100 µg/ml; chloramphenicol (Cm), 7 µg/ml (*Lm*) or 20 µg/ml (*E. coli*); erythromycin (Ery), 5 µg/ml. For the selection of pPL2 integrants following conjugation with S17-1, colistin sulfate (Col) and nalidixic acid (Nax) were used at 10 and 50 µg/ml, respectively.

II.4.2. Construction of strains expressing FLAG-tagged cell wall-binding domains of GW proteins

A master plasmid vector derived from pPL2 (Lauer *et al.* 2002) was constructed to allow the expression and secretion of N-terminally FLAG-tagged proteins in *Lm* strains from chromosome-integrated single-copy genes. A 270-bp DNA fragment comprising the *ami* promoter (P_{ami}) and Ami signal peptide (residues 1–30) sequences followed by a FLAG tag sequence was produced by PCR using *Lm* EGD-e genomic DNA as template and primers 1–2 (Table 2). After purification, the PCR fragment was digested and cloned between the *Sal*I and *Pst*I sites of pPL2, yielding pDC426 (Fig. 2A). This plasmid was then used to generate derivative constructs, each containing the GW repeat domain of one of the nine GW proteins encoded in the *Lm* genome (Cabanes *et al.* 2002). PCR fragments comprising the GW repeat domain of InlB ($InlB_{GW}$, 721 bp), Auto ($Auto_{GW}$, 1012 bp), Lmo1215 ($Lmo1215_{GW}$, 256 bp), Lmo1216 ($Lmo1216_{GW}$, 445 bp), Lmo1521 ($Lmo1521_{GW}$, 607 bp), Lmo2203 ($Lmo2203_{GW}$, 523 bp), Ami (Ami_{GW}, 1993 bp), Lmo2591 ($Lmo2591_{GW}$, 979 bp) and Lmo2713 ($Lmo2713_{GW}$, 253 bp) were produced from *Lm* EGD-e genomic DNA using, respectively, the primer pairs 3–4, 5–6, 7–8, 9–10, 11–12, 13–14, 15–16, 17–18 and 19–20 (Table 2). After purification, fragments were digested with the appropriate restriction enzymes

(Roche Applied Sciences) and cloned between the *Pst*I and *Not*I sites of pDC426, to yield pDC481, pDC459, pDC460, pDC461, pDC480, pDC462, pDC440, pDC463 and pDC464. Each of the nine plasmid constructs were introduced into *E. coli* S17-1 and transferred to both wild-type EGD-e and Δ*rmlACBD* strains by conjugation on BHI agar. Transconjugant clones were selected in BHI–Cm/Col/Nax and chromosomal integration of the plasmids confirmed by PCR with primers 21 and 22 (Table 2). Plasmid constructs were confirmed by both PCR and DNA sequencing.

II.4.3. Autolysis assay

The autolytic activity of *Lm* strains was monitored *in vitro*. Bacterial cultures grown to the exponential phase (OD_{600}=1.0) were centrifuged and the pelleted cells were washed with ice-cold bi-distilled water and resuspended in 50 mM glycine buffer (pH 8.0) to a final OD_{600} value of 1.0. Bacterial suspensions were incubated at 37 ºC with shaking and the autolytic activity was measured throughout time as the percentage of OD_{600} decrease relative to the initial value. For each time point, values were presented as mean ± standard deviation of three independent experiments.

II.4.4. Analysis of *Lm* surface and secreted protein extracts

Extraction of non-covalently surface-associated and secreted *Lm* proteins was performed as described (Braun *et al.* 1997, Cabanes *et al.* 2004), with minor changes. Twenty-milliliter samples of *Lm* cultures grown to the exponential phase (OD_{600}=0.8) were centrifuged (4,500 rpm, 15 min, 4 ºC) and the bacterial pellet and culture supernatant recovered for further processing. Bacteria were washed with ice-cold PBS, resuspended in 1.5 ml of a 2% SDS solution in PBS and incubated for 30 min at 37 ºC, to allow the extraction of non-covalently associated surface proteins. After centrifugation (15,000 rpm, 1 min), the recovered supernatant was filtered (0.22 µm) inactivated concentrated/dialysed against PBS in Vivaspin 4 (10-kDa cutoff) concentrators (Sartorius Stedim). Culture supernatants were filtered (0.22 µm) and treated with a protease inhibitor cocktail

mix (cOmplete, Roche Applied Sciences) before precipitation of proteins with the sequential addition of 0.2 mg/ml of sodium deoxycholate (30 min, 4 ºC) and 6% (v/v) TCA (overnight, 4 ºC). Proteins were collected by centrifugation (12,000 rpm, 15 min, 4 ºC) and washed twice with cold acetone. The pellet was air-dried and resuspended in 20 mM Tris-HCl buffer (pH 7.4) to a final volume of 200 µl. Proteins extracts were quantified (A_{280}) in a NanoDrop 1000 spectrophotometer (Thermo Scientific).

Protein extracts were analyzed by SDS-PAGE in an 10% (v/v) polyacrylamide gel and stained with Coomassie Brilliant Blue or transferred (Trans-Blot Turbo Transfer System, Bio-Rad Laboratories) onto a nitrocellulose membrane and probed with mouse monoclonal anti-FLAG (clone M2, Sigma-Aldrich), diluted 1:1000 (for surface protein extracts) or 1:250 (for secreted protein extracts); mouse monoclonal anti-InlB (H15.1, Braun *et al.* 1999), diluted 1:1000; rabbit polyclonal anti-Ami antiserum (R5, kind gift from Pascale Cossart), diluted 1:2000; rabbit polyclonal anti-*Lm* GAPDH ($GAPDH_{Lm}$, Abgent), diluted 1:2000 (for surface protein extracts) or 1:1000 (for secreted protein extracts); and then with anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (P.A.R.I.S Biotech), diluted 1:2000. Immunolabeled proteins were detected by chemiluminescence using Western Blotting Substrate kit (Pierce).

II.4.5. Cell line infection assays

Human colorectal adenocarcinoma Caco-2 (ATCC HTB-37™) and cervix adenocarcinoma HeLa (ATCC CCL-2™) cell lines were propagated at 37 ºC (7% CO_2) in Eagle's Minimum Essential Medium (EMEM) supplemented with 20% fetal bovine serum (FBS), 1% sodium pyruvate and 1% non-essential amino acids, and in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Lonza). To assess bacterial adhesion to host cells, confluent cell monolayers (~2×10^5/well) were inoculated for 30 min at 37 ºC (7% CO_2) with exponential-phase bacteria (OD_{600} 0.6) at a multiplicity of infection (MOI) of 75 bacteria/cell in cell culture medium. After removing the inoculum, cells were washed three times with warm medium to remove weakly associated bacteria and lysed with 1 ml of cold 0.2% Triton X-100. Ten-fold serial dilutions were plated in BHI agar and

incubated overnight at 37 ºC to allow quantification of cell-adhering bacteria. To assess bacterial invasion of host cells, confluent monolayers were inoculated for 1 hour at 37 ºC (7% CO_2) with exponential-phase bacteria (OD_{600} 0.6) at a MOI 75. After removing the inoculum, cells were incubated for 1.5 hours at 37 ºC (7% CO_2) with 20 µg/ml gentamicin, to kill extracellular bacteria. Cells were then treated as before for quantification of intracellular viable bacteria. Each condition was assayed in triplicate in at least three independent assays.

II.5. Tables

Table 1. Plasmids and bacterial strains

Plasmid or strain	Code	Relevant characteristics	Source
Plasmids			
pPL2		*L. monocytogenes* phage-derived site-specific integration vector; Cm^r	Lauer *et al.* 2002
pPL2(P_{ami}-Ami_{SP}-FLAG)	pDC426	pPL2 carrying a DNA fragment containing the *ami* promoter and Ami signal peptide (SP, residues 1–30)-encoding sequences, followed by a FLAG tag (DYKDDDDK)-encoding sequence; Cm^r	This study
pDC426($InlB_{GW}$)	pDC481	pDC426 carrying a DNA fragment encoding the InlB GW repeat domain (residues 399–630); Cm^r	This study
pDC426($Auto_{GW}$)	pDC459	pDC426 carrying a DNA fragment encoding the Auto GW repeat domain (residues 244–572); Cm^r	This study
pDC426($Lmo1215_{GW}$)	pDC460	pDC426 carrying a DNA fragment encoding the Lmo1215 GW repeat domain (residues 213–283); Cm^r	This study
pDC426($Lmo1216_{GW}$)	pDC461	pDC426 carrying a DNA fragment encoding the Lmo1216 GW repeat domain (residues 189–265); Cm^r	This study
pDC426($Lmo1521_{GW}$)	pDC480	pDC426 carrying a DNA fragment encoding the Lmo1521 GW repeat domain (residues 37–231); Cm^r	This study
pDC426($Lmo2203_{GW}$)	pDC462	pDC426 carrying a DNA fragment encoding the Lmo2203 GW repeat domain (residues 210–375); Cm^r	This study
pDC426(Ami_{GW})	pDC440	pDC426 carrying a DNA fragment encoding the Ami GW repeat domain (residues 262–917); Cm^r	This study
pDC426($Lmo2591_{GW}$)	pDC463	pDC426 carrying a DNA fragment encoding the Ami GW repeat domain (residues 191–508); Cm^r	This study
pDC426($Lmo2713_{GW}$)	pDC464	pDC426 carrying a DNA fragment encoding the Ami GW repeat domain (residues 33–109); Cm^r	This study
***E. coli* strains**			
DH5α		Cloning host strain; F^- Φ80*lac*ZΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Life Technologies
S17-1		Conjugative donor strain; *rec*A *pro hsdR* RP4-2-Tc::Mu-Km::Tn7	Simon *et al.* 1983
***L. monocytogenes* strains**			
EGD-e		wild type; serotype 1/2a	Glaser *et al.* 2001
EGD-e Δ*rmlACBD*	DC307	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant	Carvalho *et al.* 2015
EGD-e Δ*rmlACBD*::pPL2(*rmlACBD*)	DC367	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant complemented with pPL2(*rmlACBD*) (pDC313); Cm^r	Carvalho *et al.* 2015
EGD-e::pDC426($InlB_{GW}$)	DC484	EGD-e carrying pDC426($InlB_{GW}$) (pDC481); Cm^r	This study

(Continued from previous page)

Plasmid or strain	Code	Relevant characteristics	Source
***L. monocytogenes* strains**			
EGD-e Δ*rmlACBD*::pDC426($InlB_{GW}$)		EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426($InlB_{GW}$) (pDC481); Cm^r	This study
EGD-e::pDC426($Auto_{GW}$)	DC475	EGD-e carrying pDC426($Auto_{GW}$) (pDC459); Cm^r	This study
EGD-e Δ*rmlACBD*::pDC426($Auto_{GW}$)	DC467	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426($Auto_{GW}$) (pDC459); Cm^r	This study
EGD-e::pDC426($Lmo1215_{GW}$)	DC476	EGD-e carrying pDC426($Lmo1215_{GW}$) (pDC460); Cm^r	This study
EGD-e Δ*rmlACBD*::pDC426($Lmo1215_{GW}$)	DC468	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426($Lmo1215_{GW}$) (pDC460); Cm^r	This study
EGD-e::pDC426($Lmo1216_{GW}$)	DC477	EGD-e carrying pDC426($Lmo1216_{GW}$) (pDC461); Cm^r	This study
EGD-e Δ*rmlACBD*::pDC426($Lmo1216_{GW}$)	DC469	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426($Lmo1216_{GW}$) (pDC461); Cm^r	This study
EGD-e::pDC426($Lmo1521_{GW}$)	DC482	EGD-e carrying pDC426($Lmo1521_{GW}$) (pDC480); Cm^r	This study
EGD-e Δ*rmlACBD*::pDC426($Lmo1521_{GW}$)	DC483	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426($Lmo1521_{GW}$) (pDC480); Cm^r	This study
EGD-e::pDC426($Lmo2203_{GW}$)	DC478	EGD-e carrying pDC426($Lmo2203_{GW}$) (pDC462); Cm^r	This study
EGD-e Δ*rmlACBD*::pDC426($Lmo2203_{GW}$)	DC470	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426($Lmo2203_{GW}$) (pDC462); Cm^r	This study
EGD-e::pDC426(Ami_{GW})	DC445	EGD-e carrying pDC426(Ami_{GW}) (pDC440); Cm^r	This study
EGD-e Δ*rmlACBD*::pDC426(Ami_{GW})	DC446	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426(Ami_{GW}) (pDC440); Cm^r	This study
EGD-e::pDC426($Lmo2591_{GW}$)	DC479	EGD-e carrying pDC426($Lmo2591_{GW}$) (pDC463); Cm^r	This study
EGD-e Δ*rmlACBD*::pDC426($Lmo2591_{GW}$)	DC471	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426($Lmo2591_{GW}$) (pDC463); Cm^r	This study
EGD-e::pDC426($Lmo2713_{GW}$)	DC485	EGD-e carrying pDC426($Lmo2713_{GW}$) (pDC464); Cm^r	This study
EGD-e Δ*rmlACBD*::pDC426($Lmo2713_{GW}$)	DC472	EGD-e *rmlACBD* (*lmo1081–4*) deletion mutant carrying pDC426($Lmo2713_{GW}$) (pDC464); Cm^r	This study

Table 2. Primers

#	Name	Sequence (5' to 3')[a]
1	P_{ami}-*Sal*I-F	AGCGTCGACTTTACAGAAAAAAACATACG
2	Ami_{SP}-FLAG-*Pst*I-R	ATTCTGCAGC***CTTGTCATCGTCATCCTTGTAATC***AGCACTTGCTTTTTCT GTAATCATAGTAGCGGAGG
3	$InlB_{GW}$-*Pst*I-F	ATCCTGCAGGCTTAACCCGCTATGTCAAATATATTCG
4	$InlB_{GW}$-*Not*I-R	AGAGCGGCCGCTTATTTCTGTGCCCTTAAATTAGCTGC
5	$Auto_{GW}$-*Nsi*I-F	ATCATGCATGCACTCTGTACGATACGATTAAACAAC
6	$Auto_{GW}$-*Not*I-R	AGAGCGGCCGCTTAATATTTAAATGCTTTTTTGTCCATCC
7	$Lmo1215_{GW}$-*Nsi*I-F	ATCATGCATGCTATGATACAAAATCAACTGGC
8	$Lmo1215_{GW}$-*Not*I-R	AGAGCGGCCGCCTACTTTTTATCGATTAATTCTTGTTTCAC
9	$Lmo1216_{GW}$-*Pst*I-F	ATCCTGCAGGCTCAGATAAAGCAATTAAGGCTACTGG
10	$Lmo1216_{GW}$-*Not*I-R	AGAGCGGCCGCTTACCAGATGATGGATTGAACGTTC
11	$Lmo1521_{GW}$-*Pst*I-F	ATCCTGCAGGCGAAGTCTTAAATGTCCGTAGCGGTCC
12	$Lmo1521_{GW}$-*Not*I-R	AGAGCGGCCGCTTAATCGACTACCCAGTTAGCTACATAACC
13	$Lmo2203_{GW}$-*Pst*I-F	ATCCTGCAGGCTCAACAGAAAACCAATTAGCATACG
14	$Lmo2203_{GW}$-*Not*I-R	AGAGCGGCCGCTTATTTTACTTCTAACGCTTTATTAGG
15	Ami_{GW}-*Pst*I-F	ATCCTGCAGGTTTGATTAACGAAAAATATAAAGCAATGC
16	Ami_{GW}-*Not*I-R	TTTGCGGCCGCTTATTGCTTTTTAGCACTTAGG
17	$Lmo2591_{GW}$-*Pst*I-F	ATCCTGCAGGCTCTAATCAATCCTTAGCAGCAATTGG
18	$Lmo2591_{GW}$-*Not*I-R	AGAGCGGCCGCTTAACTATTGATGTCGATAGCTTTAATCC
19	$Lmo2713_{GW}$-*Nsi*I-F	ATCATGCATGCTATGACAAAGCAGTTAATCTAAAAGG
20	$Lmo2713_{GW}$-*Not*I-R	AGAGCGGCCGCTTATGTAAAGGCACGTGAGTCAATCC
21	PL95	ACATAATCAGTCCAAAGTAGATGC
22	PL102	TATCAGACCTAACCCAAACCTTCC

[a] Restriction sites underlined. FLAG tag nucleotide sequence in bolded and italicized letters.

CHAPTER IV

GENERAL DISCUSSION

The results presented in this thesis contributed to an improved understanding of the importance of WTAs and WTA glycosylation mechanisms in processes relevant for bacterial physiology, virulence and immune evasion. In particular, we have characterized the role of WTA decoration with L-rhamnose in different aspects of *Lm* biology (Fig. 28). We showed that WTA L-rhamnosylation in this organism is encoded in a single chromosomal operon containing genes for the enzymatic biosynthesis and appendage of L-rhamnose to nascent WTA polymers. We revealed that this modification is required to promote resistance and favor bacterial survival when challenged by cationic AMPs. Supported by biochemical and microscopic data, we proposed a mechanism that links AMP resistance to a WTA L-rhamnosylation-promoted reduction of the *Lm* cell wall permeability to this type of molecules. In addition, we demonstrated that WTA L-rhamnosylation ensures physiological levels of autolytic activity and supports host cell invasion. Importantly, we revealed that these contributions appear to be accomplished indirectly through the role of L-rhamnosylated WTAs in the efficient surface anchoring of GW proteins that do have an active part in these processes. Finally, we firmly established the dependence of *Lm* pathogenesis on this particular WTA tailoring mechanism.

Lm strains are distributed across several serotypes, each represented by unique cell surface antigens (Seeliger and Höhne 1979, Seeliger and Langer 1989, Gorski 2008). Among these, WTAs are prominent serotype markers due to the highly diverse nature and organization of their backbone structure and substituent groups (Kamisango *et al.* 1983, Fiedler *et al.* 1984, Fujii *et al.* 1985, Uchikawa *et al.* 1986a). Serogroup 1/2 strains are the only ones to display L-rhamnose as a side-chain group. Although strains from serogroups 3 and 7 also possess the biosynthetic *rmlABCD* genes, internal point mutations have rendered the pathway non-functional (Eugster *et al.* 2015). In addition to the serogroup-specific substituent L-rhamnose, serogroup 1/2 WTAs are decorated with Glc*N*Ac (Kamisango *et al.* 1983), which is present in other serotypes (Fiedler *et al.* 1988). In this work, however, we have only addressed specifically the role of WTA L-rhamnosylation in different biological processes of *Lm*. Many, if not all, of these processes are extensive to other *Lm* serotypes, which contain differentially structured and/or substituted WTA backbones. Therefore, a transversal study

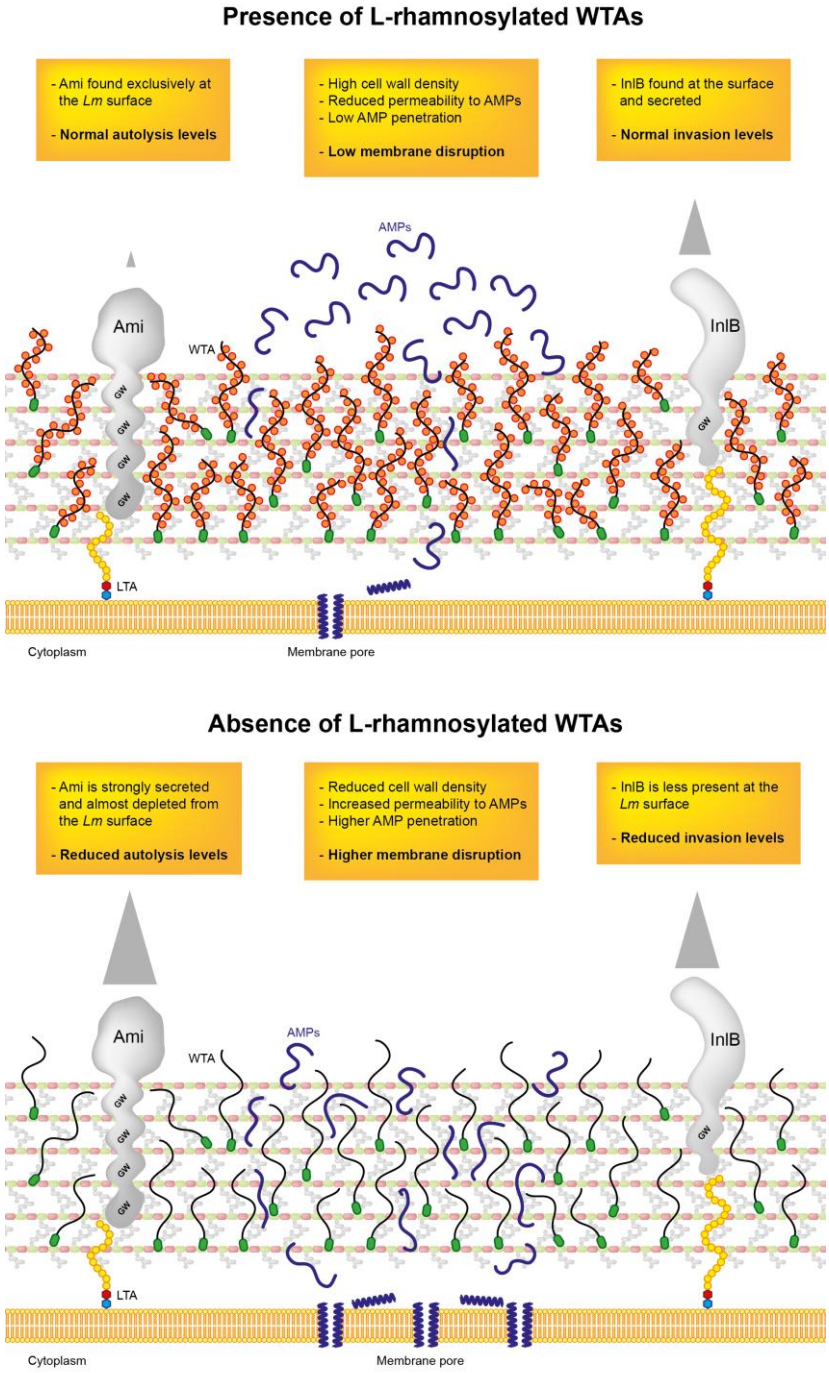

Fig. 28. Schematic representation of the proposed impact of WTA L-rhamnosylation in different aspects of *Lm* biology. This work has shown that this WTA modification promotes *Lm* resistance to AMPs by turning the cell wall less permeable to their penetration, thus delaying their contact with the plasma membrane. In addition, L-rhamnosylated WTAs contribute to the surface anchoring of some GW proteins, such as Ami and InlB, which are required to ensure optimal levels of autolysis and invasion of host cells. For simplicity, WTAs are represented only with L-rhamnose as substituent group, while only the LTA backbone is shown. Gray arrowheads represent the intensity of Ami/InlB secretion in each condition.

aimed at evaluating and comparing the biological contributions of glycosyl substituents in the many *Lm* serotypes should provide a more comprehensive perspective on the overall role of WTA substitution in *Lm* biology.

While characterizing the *Lm rmlACBD* locus, we came across *rmlT* (*lmo1080*), a gene located in the same operon and that encodes a protein with predicted glycosyltransferase activity. Biochemical analyses of the WTAs from an *Lm* mutant strain where this gene was inactivated confirmed the requirement of RmlT for the transfer of L-rhamnose from its cytoplasmic pool to the WTA backbone. Bioinformatic prediction of its subcellular location indicates RmlT as a cytoplasmic protein, which is in agreement with studies indicating that WTA glycosylation, unlike D-alanylation, occurs in this compartment prior to WTA export (Brown *et al.* 2013). Nevertheless, functional characterization of RmlT is necessary to definitively validate its WTA L-rhamnosyltransferase activity and localization. Such studies would include, for instance, *in vitro* activity and specificity assays, using purified recombinant RmlT variants in the presence of L-rhamnose or other glycosyl donor substrates and WTAs or similar acceptor molecules.

Bacterial autolysis was another physiological process for which we determined a dependence on *Lm* WTA L-rhamnosylation. We showed that in its absence, *Lm* displayed a lower rate of self-degradation. Further investigation revealed that the proportion of the autolytic enzyme Ami associated with the *Lm* surface was sharply diminished because the protein was not being properly anchored to the bacterial cell envelope. We postulated that these two findings are correlated, but this requires confirmation. For instance, we should not discard variations in the levels of other autolytic proteins that were not perceivable from the SDS-PAGE gel containing surface protein extracts. Also, zymographic analysis of these extracts should be able to expose WTA L-rhamnosylation-dependent changes in the activity of autolysins whose total protein levels remained unaffected. The autolytic profile of an *Lm* Δ*ami* strain should provide indications regarding the weight of the contribution of surface-associated Ami towards the overall autolytic process, enabling us to draw a more accurate conclusion about a link between reduced surface-associated Ami levels and reduced bacterial lysis. Alternatively, considering that our results showed that the GW domain of Auto ($Auto_{GW}$) is stably anchored to the *Lm* surface regardless of the WTA L-rhamnosylation status, we could analyze the autolytic profile of a WTA L-rhamnosylation-deficient strain mutated in the *ami* locus so as to express a

chimera protein containing the N-terminal catalytic domain of Ami and $Auto_{GW}$ in the C-terminus. We would expect this strain to be able to properly attach Ami to its cell surface and thus behave like a wild type *Lm*.

Our study of the GW domain- and WTA L-rhamnosylation-dependent surface anchoring of *Lm* GW proteins was the first to provide some information about many of these proteins. Indeed, apart from InlB, Ami and Auto (Lingnau *et al.* 1995, Braun *et al.* 1997, Cabanes *et al.* 2004), nothing was known about the localization of the remaining GW proteins. Interestingly, while Ami_{GW} appears to be completely displaced from the bacterial surface in the absence of L-rhamnosylated WTAs, the native full-length protein still preserves some surface-associated levels, as analyzed by SDS-PAGE. This suggests that the N-terminal catalytic domain of Ami may partly contribute to surface attachment. Braun and colleagues showed that the strength of the interaction between GW proteins and the *Lm* surface was correlated with the number of GW repeats (Braun *et al.* 1997). Our results in wild type bacteria agree with this observation: the longest GW domains – Ami_{GW} (8 repeats) and $Auto_{GW}$ (~4 repeats) – are exclusively surface-associated, while smaller ones – InlB (~3 repeats) > $Lmo1521_{GW}$/$Lmo2203_{GW}$/$Lmo2713_{GW}$ (2 repeats) > $Lmo1215_{GW}$/$Lmo1216_{GW}$ (1 repeat) – are increasingly delocalized to the secreted fraction. However, this trend changes in the absence of WTA L-rhamnosylation, with Ami_{GW} being completely secreted whereas $Auto_{GW}$ remains at the surface. A striking case is observed with $Lmo2591_{GW}$, which is fully secreted irrespective of the WTA L-rhamnosylation status. However, $Lmo2591_{GW}$ is also similar in molecular size and number of GW repeats to $Auto_{GW}$, which is exclusively found at the surface in both strains. These antagonistic behaviors suggest that surface anchoring properties may also be modulated by the amino acid sequence of GW modules, particularly by the non-conserved regions specific to each GW protein (Braun *et al.* 1997, Cabanes *et al.* 2002, Marino *et al.* 2002). Structural studies involving these apparently similar GW domains ought to provide elucidating data regarding the veracity of this hypothesis.

As Ami, the surface levels of InlB were shown to decrease in bacteria lacking L-rhamnosylated WTAs, due to inefficient protein anchoring. This perturbation in the surface localization of a key *Lm* invasin prompted us to assess the cell invasive properties of these mutant bacteria. Our results showed a significant drop

in intracellular *Lm* numbers, confirming a defect in the invasion of epithelial cells. It would be interesting to verify if this attenuated phenotype is also observed in other cell types, such as parenchymal (e.g. hepatocytes) or endothelial cells. To try to confirm whether the decreased levels of the surface-associated InlB form are responsible for the attenuated invasion phenotype, a similar chimera protein strategy could be also employed. In this case, a WTA L-rhamnosylation mutant strain would express the N-terminal functional domain of InlB, containing the c-Met-binding LRR region (Shen *et al.* 2000), fused to the $Auto_{GW}$. This fusion would hypothetically keep InlB activity segregated to the *Lm* surface. However, in normal conditions, InlB is partly secreted (Braun *et al.* 1997) and this soluble form was reported to contribute to the optimization of InlB-mediated *Lm* internalization by host cells (Jonquières *et al.* 2001). Still, a chimeric protein containing the LRR and inter-repeat regions of InlB fused to the C-terminal region of the staphylococcal protein A (SPA) – which mediates a stable covalent association with the cell wall – was able to greatly potentiate (100-fold) the invasion of Vero cells by non-invasive *L. innocua* (Braun *et al.* 1999).

In part I of the results, the requirement of WTA L-rhamnosylation for optimal *Lm* virulence levels was demonstrated in a mouse infection model. Although these infection assays suggest that a major part of the *in vivo* attenuation of *Lm* WTA L-rhamnosylation mutants is due to the impaired capacity to resist to host-produced AMPs, it cannot be ruled out that a part of this attenuated phenotype is a reflection of the undermined ability of these bacteria to invade cells, as shown in part II of the results. A way to address this possibility would be to perform infection assays on wild-type mice with a WTA L-rhamnosylation mutant strain expressing InlB solely as a chimera with $Auto_{GW}$ or the C-terminal region SPA (to dissociate its anchoring mechanism from the WTA L-rhamnosylation status) and observe whether virulence levels are increased relative to an isogenic strain expressing native InlB or even comparable to those of a wild type *Lm*.

In conclusion, our findings have expanded the current knowledge on WTAs, but most importantly, have raised the status and influence of WTA glycosylation mechanisms in overall bacterial biology to a previously unrecognized level. Particularly significant – and the base of all these newly identified contributions – is the newfound role of WTA L-rhamnosyl substituents in assisting the non-covalent

binding of bacterial surface proteins with common anchoring motif domains. Further work will be necessary to determine if this supporting mechanism is exclusively dependent on WTA L-rhamnosylation or if it is broadly guided by WTA glycosyl substituents in general. Most importantly, additional investigation should focus on understanding the molecular details governing these WTA glycosylation-dependent protein interactions with the bacterial cell envelope.

CHAPTER V

REFERENCES

Abachin, E, C Poyart, E Pellegrini, E Milohanic, F Fiedler*, et al.* (2002). "Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*." *Mol Microbiol* **43**: 1-14.

Abi Khattar, Z, A Rejasse, D Destoumieux-Garzon, JM Escoubas, V Sanchis*, et al.* (2009). "The dlt operon of *Bacillus cereus* is required for resistance to cationic antimicrobial peptides and for virulence in insects." *J Bacteriol* **191**(22): 7063-7073.

Aguirre-Ramírez, M, G Medina, A González-Valdez, V Grosso-Becerra and G Soberón-Chávez (2012). "The *Pseudomonas aeruginosa rmlBDAC* operon, encoding dTDP-L-rhamnose biosynthetic enzymes, is regulated by the quorum-sensing transcriptional regulator RhlR and the alternative sigma factor σS." *Microbiology (Reading, England)* **158**: 908-916.

Allerberger, F and M Wagner (2010). "Listeriosis: a resurgent foodborne infection." *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **16**: 16-23.

Allgaier, H, G Jung, RG Werner, U Schneider and H Zahner (1986). "Epidermin: sequencing of a heterodetic tetracyclic 21-peptide amide antibiotic." *Eur J Biochem* **160**(1): 9-22.

Allignet, J, S Aubert, KG Dyke and N El Solh (2001). "*Staphylococcus caprae* strains carry determinants known to be involved in pathogenicity: a gene encoding an autolysin-binding fibronectin and the ica operon involved in biofilm formation." *Infect Immun* **69**: 712-718.

Alonzo, F, PD McMullen and NE Freitag (2011). "Actin polymerization drives septation of *Listeria monocytogenes namA* hydrolase mutants, demonstrating host correction of a bacterial defect." *Infect Immun* **79**: 1458-1470.

Alonzo, F, GC Port, M Cao and NE Freitag (2009). "The posttranslocation chaperone PrsA2 contributes to multiple facets of *Listeria monocytogenes* pathogenesis." *Infect Immun* **77**: 2612-2623.

Alvarez-Dominguez, C, Ja Vazquez-Boland, E Carrasco-Marin, P Lopez-Mato and F Leyva-Cobian (1997). "Host cell heparan sulfate proteoglycans mediate attachment and entry of *Listeria monocytogenes*, and the listerial surface protein ActA is involved in heparan sulfate receptor recognition." *Infect Immun* **65**: 78-88.

Andra, J, T Goldmann, CM Ernst, A Peschel and T Gutsmann (2011). "Multiple peptide resistance factor (MprF)-mediated Resistance of *Staphylococcus aureus* against antimicrobial peptides coincides with a modulated peptide interaction with artificial membranes comprising lysyl-phosphatidylglycerol." *J Biol Chem* **286**(21): 18692-18700.

Araki, Y and E Ito (1989). "Linkage units in cell walls of gram-positive bacteria." *Critical reviews in microbiology* **17**(2): 121-135.

Archibald, AR, J Baddiley and S Heptinstall (1973). "The alanine ester content and magnesium binding capacity of walls of *Staphylococcus aureus* H grown at different pH values." *Biochimica et Biophysica Acta* **291**(3): 629-634.

Armstrong, JJ, J Baddiley, JG Buchanan, B Carss and GR Greenberg (1958). "Isolation and Structure of Ribitol Phosphate Derivatives (Teichoic Acids) from Bacterial Cell Walls." *J Chem Soc*, Doi 10.1039/Jr9580004344(Dec): 4344-4354.

Arnaud, M, A Chastanet and M Débarbouillé (2004). "New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria." *Applied and environmental microbiology* **70**: 6887-6891.

Asano, K, I Kakizaki and A Nakane (2012). "Interaction of *Listeria monocytogenes* autolysin amidase with glycosaminoglycans promotes listerial adhesion to mouse hepatocytes." *Biochimie* **94**: 1291-1299.

Atilano, ML, PM Pereira, J Yates, P Reed, H Veiga*, et al.* (2010). "Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*." *Proceedings of the National Academy of Sciences of the United States of America* **107**: 18991-18996.

Aubry, C, C Goulard, M-A Nahori, N Cayet, J Decalf*, et al.* (2011). "OatA, a peptidoglycan O-acetyltransferase involved in *Listeria monocytogenes* immune escape, is critical for virulence." *The Journal of infectious diseases* **204**: 731-740.

Auerbuch, V, JJ Loureiro, FB Gertler, JA Theriot and DA Portnoy (2003). "Ena/VASP proteins contribute to *Listeria monocytogenes* pathogenesis by controlling temporal and spatial persistence of bacterial actin-based motility." *Mol Microbiol* **49**(5): 1361-1375.

Autret, N, I Dubail, P Trieu-Cuot, P Berche and A Charbit (2001). "Identification of new genes involved in the virulence of *Listeria monocytogenes* by signature-tagged transposon mutagenesis." *Infect Immun* **69**: 2054-2065.

Ayabe, T, DP Satchell, CL Wilson, WC Parks, ME Selsted*, et al.* (2000). "Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria." *Nature immunology* **1**(2): 113-118.

Babasaki, K, T Takao, Y Shimonishi and K Kurahashi (1985). "Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis." *Journal of biochemistry* **98**(3): 585-603.

Babu, MM, ML Priya, AT Selvan, M Madera, J Gough*, et al.* (2006). "A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins." *J Bacteriol* **188**: 2761-2773.

Baddiley, J (1970). "Structure, Biosynthesis, and Function of Teichoic Acids." *Accounts Chem Res* **3**(3): 98-&.

Baddiley, J (1972). "Teichoic acids in cell walls and membranes of bacteria." *Essays in biochemistry* **8**: 35-77.

Baddiley, J, JG Buchanan, FE Hardy, RO Martin, UL Rajbhandary*, et al.* (1961). "The structure of the ribitol teichoic acid of *Staphylococcus aureus* H." *Biochim Biophys Acta* **52**: 406-407.

Banerjee, M, J Copp, D Vuga, M Marino, T Chapman*, et al.* (2004). "GW domains of the *Listeria monocytogenes* invasion protein InlB are required for potentiation of Met activation." *Mol Microbiol* **52**: 257-271.

Bateman, A and ND Rawlings (2003). "The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases." *Trends Biochem Sci* **28**: 234-237.

Baumgärtner, M, U Kärst, B Gerstel, M Loessner, J Wehland*, et al.* (2007). "Inactivation of Lgt allows systematic characterization of lipoproteins from *Listeria monocytogenes*." *J Bacteriol* **189**: 313-324.

Beauregard, KE, KD Lee, RJ Collier and JA Swanson (1997). "pH-dependent perforation of macrophage phagosomes by listeriolysin O from *Listeria monocytogenes*." *The Journal of experimental medicine* **186**(7): 1159-1163.

Belas, R, J Manos and R Suvanasuthi (2004). "*Proteus mirabilis* ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides." *Infect Immun* **72**(9): 5159-5167.

Bera, A, R Biswas, S Herbert and F Gotz (2006). "The presence of peptidoglycan O-acetyltransferase in various staphylococcal species correlates with lysozyme resistance and pathogenicity." *Infect Immun* **74**(8): 4598-4604.

Bera, A, R Biswas, S Herbert, E Kulauzovic, C Weidenmaier*, et al.* (2007). "Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*." *J Bacteriol* **189**: 280-283.

Bera, A, S Herbert, A Jakob, W Vollmer and F Götz (2005). "Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*." *Mol Microbiol* **55**: 778-787.

Bertsch, D, J Rau, MR Eugster, MC Haug, PA Lawson*, et al.* (2013). "*Listeria fleischmannii* sp nov., isolated from cheese." *Int J Syst Evol Micr* **63**: 526-532.

Beven, L, O Helluin, G Molle, H Duclohier and H Wroblewski (1999). "Correlation between anti-bacterial activity and pore sizes of two classes of voltage-dependent channel-forming peptides." *Biochim Biophys Acta* **1421**(1): 53-63.

Bevins, CL (1994). "Antimicrobial peptides as agents of mucosal immunity." *Ciba Foundation symposium* **186**: 250-260; discussion 261-259.

Bieler, S, F Silva, C Soto and D Belin (2006). "Bactericidal activity of both secreted and nonsecreted microcin e492 requires the mannose permease." *J Bacteriol* **188**(20): 7049-7061.

Bierbaum, G and HG Sahl (1987). "Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase." *J Bacteriol* **169**(12): 5452-5458.

Bierne, H and P Cossart (2007). "*Listeria monocytogenes* surface proteins: from genome predictions to function." *Microbiology and molecular biology reviews : MMBR* **71**: 377-397.

Bierne, H, C Garandeau, MG Pucciarelli, C Sabet, S Newton*, et al.* (2004). "Sortase B, a new class of sortase in *Listeria monocytogenes*." *J Bacteriol* **186**: 1972-1982.

Bierne, H, C Sabet, N Personnic and P Cossart (2007). "Internalins: a complex family of leucine-rich repeat-containing proteins in *Listeria monocytogenes*." *Microbes and infection / Institut Pasteur* **9**: 1156-1166.

Birkeland, NK (1994). "Cloning, molecular characterization, and expression of the genes encoding the lytic functions of lactococcal bacteriophage phi LC3: a dual lysis system of modular design." *Canadian journal of microbiology* **40**: 658-665.

Biswas, R, RE Martinez, N Gohring, M Schlag, M Josten*, et al.* (2012). "Proton-binding capacity of *Staphylococcus aureus* wall teichoic acid and its role in controlling autolysin activity." *PloS one* **7**(7): e41415.

Blanot, S, C Boumaila and P Berche (1999). "Intracerebral activity of antibiotics against *Listeria monocytogenes* during experimental rhombencephalitis." *Journal of Antimicrobial Chemotherapy* **44**: 565-568.

Boman, HG, B Agerberth and A Boman (1993). "Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine." *Infect Immun* **61**(7): 2978-2984.

Boneca, IG, O Dussurget, D Cabanes, M-A Nahori, S Sousa*, et al.* (2007). "A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system." *Proceedings of the National Academy of Sciences of the United States of America* **104**: 997-1002.

Borezée, E, E Pellegrini, JL Beretti and P Berche (2001). "SvpA, a novel surface virulence-associated protein required for intracellular survival of *Listeria monocytogenes*." *Microbiology (Reading, England)* **147**: 2913-2923.

Boujemaa-Paterski, R, E Gouin, G Hansen, S Samarin, C Le Clainche*, et al.* (2001). "*Listeria* protein ActA mimics WASP family proteins: It activates filament barbed end branching by Arp2/3 complex." *Biochemistry* **40**: 11390-11404.

Braun, L, S Dramsi, P Dehoux, H Bierne, G Lindahl*, et al.* (1997). "InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association." *Mol Microbiol* **25**: 285-294.

Braun, L, B Ghebrehiwet and P Cossart (2000). "gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*." *The EMBO journal* **19**: 1458-1466.

Braun, L, F Nato, B Payrastre, JC Mazié and P Cossart (1999). "The 213-amino-acid leucine-rich repeat region of the *Listeria monocytogenes* InlB protein is sufficient for entry into mammalian cells, stimulation of PI 3-kinase and membrane ruffling." *Mol Microbiol* **34**: 10-23.

Braun, L, H Ohayon and P Cossart (1998). "The InlB protein of *Listeria monocytogenes* is sufficient to promote entry into mammalian cells." *Mol Microbiol* **27**: 1077-1087.

Breton, C, L Snajdrová, C Jeanneau, J Koca and A Imberty (2006). "Structures and mechanisms of glycosyltransferases." *Glycobiology* **16**: 29R-37R.

Breukink, E and B de Kruijff (1999). "The lantibiotic nisin, a special case or not?" *Biochim Biophys Acta* **1462**(1-2): 223-234.

Brogden, Ka (2005). "Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?" *Nature reviews Microbiology* **3**: 238-250.

Brotz, H, G Bierbaum, K Leopold, PE Reynolds and HG Sahl (1998a). "The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II." *Antimicrob Agents Chemother* **42**(1): 154-160.

Brotz, H, M Josten, I Wiedemann, U Schneider, F Gotz*, et al.* (1998b). "Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics." *Mol Microbiol* **30**(2): 317-327.

Brown, S, JP Santa Maria and S Walker (2013). "Wall teichoic acids of gram-positive bacteria." *Annual review of microbiology* **67**: 313-336.

Brown, S, G Xia, LG Luhachack, J Campbell, TC Meredith*, et al.* (2012). "Methicillin resistance in *Staphylococcus aureus* requires glycosylated wall teichoic acids." *Proceedings of the National Academy of Sciences of the United States of America* **109**: 18909-18914.

Bublitz, M, L Polle, C Holland, DW Heinz, M Nimtz*, et al.* (2009). "Structural basis for autoinhibition and activation of Auto, a virulence-associated peptidoglycan hydrolase of *Listeria monocytogenes*." *Mol Microbiol* **71**(6): 1509-1522.

Buchrieser, C (2007). "Biodiversity of the species *Listeria monocytogenes* and the genus *Listeria*." *Microbes and infection / Institut Pasteur* **9**: 1147-1155.

Buchrieser, C, C Rusniok, P Garrido, T Hain, M Scortti*, et al.* (2011). "Complete Genome Sequence of the Animal Pathogen *Listeria ivanovii*, Which Provides

Insights into Host Specificities and Evolution of the Genus *Listeria*." *J Bacteriol* **193**(23): 6787-6788.

Buchrieser, C, C Rusniok, F Kunst, P Cossart and P Glaser (2003). "Comparison of the genome sequences of *Listeria monocytogenes* and *Listeria innocua*: clues for evolution and pathogenicity." *FEMS Immunology & Medical Microbiology* **35**: 207-213.

Buist, G, J Kok, KJ Leenhouts, M Dabrowska, G Venema*, et al.* (1995). "Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation." *J Bacteriol* **177**: 1554-1563.

Buist, G, A Steen, J Kok and OP Kuipers (2008). "LysM, a widely distributed protein motif for binding to (peptido)glycans." *Mol Microbiol* **68**: 838-847.

Cabanes, D, P Dehoux, O Dussurget, L Frangeul and P Cossart (2002). "Surface proteins and the pathogenic potential of *Listeria monocytogenes*." *Trends in microbiology* **10**: 238-245.

Cabanes, D, O Dussurget, P Dehoux and P Cossart (2004). "Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence." *Mol Microbiol* **51**: 1601-1614.

Cabanes, D, M Lecuit and P Cossart (2008). "Animal models of Listeria infection." *Current protocols in microbiology* **Chapter 9**: Unit9B.1.

Cabanes, D, S Sousa, A Cebriá, M Lecuit, F García-del Portillo*, et al.* (2005). "Gp96 is a receptor for a novel *Listeria monocytogenes* virulence factor, Vip, a surface protein." *The EMBO journal* **24**: 2827-2838.

Camejo, A, C Buchrieser, E Couvé, F Carvalho, O Reis*, et al.* (2009). "*In vivo* transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection." *PLoS pathogens* **5**: e1000449.

Camejo, A, F Carvalho, O Reis, E Leitão, S Sousa*, et al.* (2011). "The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle." *Virulence* **2**: 379-394.

Campos, MA, MA Vargas, V Regueiro, CM Llompart, S Alberti*, et al.* (2004). "Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides." *Infect Immun* **72**(12): 7107-7114.

Carrero, JA, B Calderon and ER Unanue (2004). "Listeriolysin O from *Listeria monocytogenes* is a lymphocyte apoptogenic molecule." *J Immunol* **172**(8): 4866-4874.

Carroll, SA, T Hain, U Technow, A Darji, P Pashalidis*, et al.* (2003). "Identification and characterization of a peptidoglycan hydrolase, MurA, of *Listeria monocytogenes*, a muramidase needed for cell separation." *J Bacteriol* **185**: 6801-6808.

Carvalho, F, ML Atilano, R Pombinho, G Covas, RL Gallo*, et al.* (2015). "L-Rhamnosylation of Listeria monocytogenes Wall Teichoic Acids Promotes Resistance to Antimicrobial Peptides by Delaying Interaction with the Membrane." *PLoS pathogens* **11**(5): e1004919.

Carvalho, F, MG Pucciarelli, FG-d Portillo, D Cabanes and P Cossart (2013). Extraction of cell wall-bound teichoic acids and surface proteins from *Listeria monocytogenes*. *In*: Delcour A. H. (ed.) *Methods in Molecular Biology*. Totowa, NJ, Humana Press. **966:** 289-308.

Carvalho, F, S Sousa and D Cabanes (2014). "How *Listeria monocytogenes* organizes its surface for virulence." *Frontiers in cellular and infection microbiology* **4**: 48.

Cascales, E, SK Buchanan, D Duché, C Kleanthous, R Lloubès*, et al.* (2007). "Colicin biology." *Microbiology and molecular biology reviews : MMBR* **71**: 158-229.

Castle, M, A Nazarian, SS Yi and P Tempst (1999). "Lethal effects of apidaecin on *Escherichia coli* involve sequential molecular interactions with diverse targets." *J Biol Chem* **274**(46): 32555-32564.

Cederlund, A, GH Gudmundsson and B Agerberth (2011). "Antimicrobial peptides important in innate immunity." *The FEBS journal* **278**: 3942-3951.

Chakraborty, T, F Ebel, E Domann, K Niebuhr, B Gerstel*, et al.* (1995). "A focal adhesion factor directly linking intracellularly motile *Listeria monocytogenes* and *Listeria ivanovii* to the actin-based cytoskeleton of mammalian cells." *The EMBO journal* **14**: 1314-1321.

Chan, DI, EJ Prenner and HJ Vogel (2006). "Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action." *Biochimica et Biophysica Acta - Biomembranes* **1758**: 1184-1202.

Chan, YGY, MB Frankel, V Dengler, O Schneewind and D Missiakas (2013). "*Staphylococcus aureus* Mutants Lacking the LytR-CpsA-Psr Family of Enzymes Release Cell Wall Teichoic Acids into the Extracellular Medium." *J Bacteriol* **195**: 4650-4659.

Chassaing, D and F Auvray (2007). "The *lmo1078* gene encoding a putative UDP-glucose pyrophosphorylase is involved in growth of *Listeria monocytogenes* at low temperature." *Fems Microbiol Lett* **275**: 31-37.

Chatterjee, AN, D Mirelman, HJ Singer and JT Park (1969). "Properties of a novel pleiotropic bacteriophage-resistant mutant of *Staphylococcus aureus* H." *J Bacteriol* **100**: 846-853.

Chatterjee, D (1997). "The mycobacterial cell wall: structure, biosynthesis and sites of drug action." *Current opinion in chemical biology* **1**: 579-588.

Chatterjee, SS, H Hossain, S Otten, C Kuenne, K Kuchmina*, et al.* (2006). "Intracellular gene expression profile of *Listeria monocytogenes*." *Infect Immun* **74**: 1323-1338.

Chavakis, T, DB Cines, JS Rhee, OD Liang, U Schubert*, et al.* (2004). "Regulation of neovascularization by human neutrophil peptides (alpha-defensins): a link between inflammation and angiogenesis." *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **18**(11): 1306-1308.

Cho, JH, BH Sung and SC Kim (2009). "Buforins: histone H2A-derived antimicrobial peptides from toad stomach." *Biochim Biophys Acta* **1788**(8): 1564-1569.

Chromek, M, I Arvidsson and D Karpman (2012). "The antimicrobial peptide cathelicidin protects mice from *Escherichia coli* O157:H7-mediated disease." *PloS one* **7**: e46476.

Clarke, AJ, H Strating and NT Blackburn (2002). Pathways for the O-Acetylation of Bacterial Cell Wall Polysaccharides. *In*: Doyle R. J. (ed.) *Glycomicrobiology*. Springer US, 10.1007/0-306-46821-2_7**:** 187-223.

Cole, AM, T Hong, LM Boo, T Nguyen, C Zhao*, et al.* (2002). "Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1." *Proceedings of the National Academy of Sciences of the United States of America* **99**(4): 1813-1818.

Collins, LV, SA Kristian, C Weidenmaier, M Faigle, KPM Van Kessel*, et al.* (2002). "*Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice." *The Journal of infectious diseases* **186**: 214-219.

Comfort, D and RT Clubb (2004). "A comparative genome analysis identifies distinct sorting pathways in gram-positive bacteria." *Infect Immun* **72**: 2710-2722.

Cossart, P and A Toledo-Arana (2008). "*Listeria monocytogenes*, a unique model in infection biology: an overview." *Microbes and infection / Institut Pasteur* **10**: 1041-1050.

Cotter, PD, C Hill and RP Ross (2005). "Bacteriocins: developing innate immunity for food." *Nature reviews Microbiology* **3**: 777-788.

Cotter, PD, RP Ross and C Hill (2013). "Bacteriocins - a viable alternative to antibiotics?" *Nature reviews Microbiology* **11**: 95-105.

Cramer, WA, JB Heymann, SL Schendel, BN Deriy, FS Cohen*, et al.* (1995). "Structure-function of the channel-forming colicins." *Annual review of biophysics and biomolecular structure* **24**: 611-641.

Cullen, TW, DK Giles, LN Wolf, C Ecobichon, IG Boneca*, et al.* (2011). "*Helicobacter pylori* versus the host: remodeling of the bacterial outer membrane is required for survival in the gastric mucosa." *PLoS pathogens* **7**(12): e1002454.

Dalbey, RE, P Wang and A Kuhn (2011). "Assembly of bacterial inner membrane proteins." *Annu Rev Biochem* **80**: 161-187.

Dalet, K, Y Cenatiempo, P Cossart, Y Hechard and C European Listeria Genome (2001). "A sigma(54)-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105." *Microbiology* **147**(Pt 12): 3263-3269.

de Jonge, BL, YS Chang, D Gage and A Tomasz (1992). "Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin-binding protein 2A." *The Journal of biological chemistry* **267**: 11248-11254.

de Leeuw, E, C Li, P Zeng, C Li, M Diepeveen-de Buin*, et al.* (2010). "Functional interaction of human neutrophil peptide-1 with the cell wall precursor lipid II." *Febs Lett* **584**(8): 1543-1548.

Decatur, AL and DA Portnoy (2000). "A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity." *Science (New York, NY)* **290**: 992-995.

Deininger, S, I Figueroa-Perez, S Sigel, A Stadelmaier, RR Schmidt*, et al.* (2007). "Use of synthetic derivatives to determine the minimal active structure of cytokine-inducing lipoteichoic acid." *Clin Vaccine Immunol* **14**(12): 1629-1633.

Deininger, S, A Stadelmaier, S von Aulock, S Morath, RR Schmidt*, et al.* (2003). "Definition of structural prerequisites for lipoteichoic acid-inducible cytokine induction by synthetic derivatives." *J Immunol* **170**(8): 4134-4138.

den Bakker, HC, S Warchocki, EM Wright, AF Allred, C Ahlstrom*, et al.* (2014). "*Listeria floridensis* sp nov., *Listeria aquatica* sp nov., *Listeria cornellensis* sp nov., *Listeria riparia* sp nov and *Listeria grandensis* sp nov., from agricultural and natural environments." *Int J Syst Evol Micr* **64**: 1882-1889.

Denny, J and J McLauchlin (2008). "Human *Listeria monocytogenes* infections in Europe--an opportunity for improved European surveillance." *Euro surveillance : bulletin europ??en sur les maladies transmissibles = European communicable disease bulletin* **13**: 1-5.

Desvaux, M, E Dumas, I Chafsey and M Hébraud (2006). "Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure." *Fems Microbiol Lett* **256**: 1-15.

Dhar, G, KF Faull and O Schneewind (2000). "Anchor structure of cell wall surface proteins in *Listeria monocytogenes*." *Biochemistry* **39**: 3725-3733.

Diamond, G, V Kaiser, J Rhodes, JP Russell and CL Bevins (2000). "Transcriptional regulation of beta-defensin gene expression in tracheal epithelial cells." *Infect Immun* **68**(1): 113-119.

Diamond, G, M Zasloff, H Eck, M Brasseur, WL Maloy*, et al.* (1991). "Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA." *Proceedings of the National Academy of Sciences of the United States of America* **88**(9): 3952-3956.

Diep, DB, M Skaugen, Z Salehian, H Holo and IF Nes (2007). "Common mechanisms of target cell recognition and immunity for class II bacteriocins." *Proceedings of the National Academy of Sciences of the United States of America* **104**(7): 2384-2389.

Disson, O and M Lecuit (2012). "Targeting of the central nervous system by *Listeria monocytogenes*." *Virulence* **3**: 213-221.

Domann, E, J Wehland, M Rohde, S Pistor, M Hartl*, et al.* (1992). "A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin." *The EMBO journal* **11**: 1981-1990.

Dortet, L, S Mostowy, A Samba-Louaka, AS Louaka, E Gouin*, et al.* (2011). "Recruitment of the major vault protein by InlK: a *Listeria monocytogenes* strategy to avoid autophagy." *PLoS pathogens* **7**: e1002168.

Doumith, M, C Cazalet, N Simoes, L Frangeul, C Jacquet*, et al.* (2004). "New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays." *Infect Immun* **72**: 1072-1083.

Dramsi, S, I Biswas, E Maguin, L Braun, P Mastroeni*, et al.* (1995). "Entry of *Listeria monocytogenes* into hepatocytes requires expression of InlB, a surface protein of the internalin multigene family." *Mol Microbiol* **16**: 251-261.

Dramsi, S, F Bourdichon, D Cabanes, M Lecuit, H Fsihi*, et al.* (2004). "FbpA, a novel multifunctional *Listeria monocytogenes* virulence factor." *Mol Microbiol* **53**: 639-649.

Dramsi, S and P Cossart (2003). "Listeriolysin O-mediated calcium influx potentiates entry of *Listeria monocytogenes* into the human Hep-2 epithelial cell line." *Infect Immun* **71**(6): 3614-3618.

Dramsi, S, P Dehoux, M Lebrun, PL Goossens and P Cossart (1997). "Identification of four new members of the internalin multigene family of *Listeria monocytogenes* EGD." *Infect Immun* **65**: 1615-1625.

Dramsi, S, S Magnet, S Davison and M Arthur (2008). "Covalent attachment of proteins to peptidoglycan." *Fems Microbiol Rev* **32**(2): 307-320.

Dupont, C and AJ Clarke (1991). "Dependence of lysozyme-catalysed solubilization of *Proteus mirabilis* peptidoglycan on the extent of O-acetylation." *Eur J Biochem* **195**(3): 763-769.

Duquesne, S and D Destoumieux-Garzón (2007). "Microcins, gene-encoded antibacterial peptides from enterobacteria." *Natural product …* **24**: 75005.

Eckert, C, M Lecerf, L Dubost, M Arthur and S Mesnage (2006). "Functional analysis of AtlA, the major N-acetylglucosaminidase of *Enterococcus faecalis*." *J Bacteriol* **188**: 8513-8519.

Ehrenstein, G and H Lecar (1977). "Electrically gated ionic channels in lipid bilayers." *Quarterly reviews of biophysics* **10**(1): 1-34.

Eijsink, VGH, M Skeie, PH Middelhoven, MB Brurberg and IF Nes (1998). "Comparative studies of class IIa bacteriocins of lactic acid bacteria." *Applied and environmental microbiology* **64**(9): 3275-3281.

Elmore, DE (2012). "Insights into buforin II membrane translocation from molecular dynamics simulations." *Peptides* **38**(2): 357-362.

Eugster, MR, MC Haug, SG Huwiler and MJ Loessner (2011). "The cell wall binding domain of *Listeria* bacteriophage endolysin PlyP35 recognizes terminal GlcNAc residues in cell wall teichoic acid." *Mol Microbiol* **81**: 1419-1432.

Eugster, MR and MJ Loessner (2012). "Wall teichoic acids restrict access of bacteriophage endolysin Ply118, Ply511, and PlyP40 cell wall binding domains to the *Listeria monocytogenes* peptidoglycan." *J Bacteriol* **194**: 6498-6506.

Eugster, MR, LS Morax, VJ Hüls, SG Huwiler, A Leclercq*, et al.* (2015). "Bacteriophage predation promotes serovar diversification in *Listeria monocytogenes*." *Mol Microbiol*, 10.1111/mmi.13009: n/a-n/a.

Faith, N, S Kathariou, Y Cheng, N Promadej, BL Neudeck*, et al.* (2009). "The role of *L. monocytogenes* serotype 4b *gtcA* in gastrointestinal listeriosis in A/J mice." *Foodborne pathogens and disease* **6**: 39-48.

Faith, NG, S Kathariou, BL Neudeck, JB Luchansky and CJ Czuprynski (2007). "A P60 mutant of *Listeria monocytogenes* is impaired in its ability to cause infection in intragastrically inoculated mice." *Microbial pathogenesis* **42**: 237-241.

Falla, TJ, DN Karunaratne and RE Hancock (1996). "Mode of action of the antimicrobial peptide indolicidin." *J Biol Chem* **271**(32): 19298-19303.

Farha, MA, A Leung, EW Sewell, MA D'Elia, SE Allison*, et al.* (2013). "Inhibition of WTA synthesis blocks the cooperative action of PBPs and sensitizes MRSA to beta-lactams." *Acs Chem Biol* **8**(1): 226-233.

Fedtke, I, D Mader, T Kohler, H Moll, G Nicholson*, et al.* (2007). "A *Staphylococcus aureus ypfP* mutant with strongly reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and autolysin activity." *Mol Microbiol* **65**(4): 1078-1091.

Fenlon, DR (1999). *Listeria monocytogenes* in the natural environment. *In*: Ryser E. T. and E. H. Marth (eds.), *Listeria, listeriosis, and food safety*. (2nd. edn.). New York, Marcel Decker**:** 21-37.

Fiedler, F (1988). "Biochemistry of the cell surface of *Listeria* strains: a locating general view." *Infection* **16 Suppl 2**: S92-97.

Fiedler, F, J Seger, A Schrettenbrunner and HP Seeliger (1984). "The biochemistry of murein and cell wall teichoic acids in the genus *Listeria*." *Syst Appl Microbiol* **5**: 360-376.

Filipe, SR, A Tomasz and P Ligoxygakis (2005). "Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway." *EMBO reports* **6**: 327-333.

Fischer, W (1994). "Lipoteichoic Acid and Lipids in the Membrane of *Staphylococcus Aureus*." *Med Microbiol Immun* **183**(2): 61-76.

Fischer, W, T Mannsfeld and G Hagen (1990). "On the Basic Structure of Poly(Glycerophosphate) Lipoteichoic Acids." *Biochem Cell Biol* **68**(1): 33-43.

Fischer, W and P Rosel (1980). "The Alanine Ester Substitution of Lipoteichoic Acid (Lta) in *Staphylococcus Aureus*." *Febs Lett* **119**(2): 224-226.

Fischer, W, P Rosel and HU Koch (1981). "Effect of alanine ester substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of *Staphylococcus aureus*." *J Bacteriol* **146**(2): 467-475.

Fischetti, VA, V Pancholi and O Schneewind (1990). "Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive cocci." *Mol Microbiol* **4**: 1603-1605.

Formstone, A, R Carballido-Lopez, P Noirot, J Errington and DJ Scheffers (2008). "Localization and interactions of teichoic acid synthetic enzymes in *Bacillus subtilis*." *J Bacteriol* **190**(5): 1812-1821.

Forster, BM, J Zemansky, Da Portnoy and H Marquis (2011). "Posttranslocation chaperone PrsA2 regulates the maturation and secretion of *Listeria monocytogenes* proprotein virulence factors." *J Bacteriol* **193**: 5961-5970.

Fox, RO, Jr. and FM Richards (1982). "A voltage-gated ion channel model inferred from the crystal structure of alamethicin at 1.5-A resolution." *Nature* **300**(5890): 325-330.

Fredenhagen, A, G Fendrich, F Märki, W Märki, J Gruner*, et al.* (1990). "Duramycins B and C, two new lanthionine containing antibiotics as inhibitors

of phospholipase A2. Structural revision of duramycin and cinnamycin." *Journal of Antibiotics* **43**(11): 1403-1412.

Fregeau Gallagher, NL, M Sailer, WP Niemczura, TT Nakashima, ME Stiles*, et al.* (1997). "Three-dimensional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria." *Biochemistry* **36**(49): 15062-15072.

Freymond, P-P, V Lazarevic, B Soldo and D Karamata (2006). "Poly(glucosyl-N-acetylgalactosamine 1-phosphate), a wall teichoic acid of *Bacillus subtilis* 168: its biosynthetic pathway and mode of attachment to peptidoglycan." *Microbiology (Reading, England)* **152**: 1709-1718.

Frick, IM, P Akesson, M Rasmussen, A Schmidtchen and L Bjorck (2003). "SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides." *J Biol Chem* **278**(19): 16561-16566.

Frirdich, E and C Whitfield (2005). "Lipopolysaccharide inner core oligosaccharide structure and outer membrane stability in human pathogens belonging to the Enterobacteriaceae." *Journal of endotoxin research* **11**: 133-144.

Fujii, H, K-i Kamisango, M Nagaoka, K-i Uchikawa, I Sekikawa*, et al.* (1985). "Structural study on teichoic acids of *Listeria monocytogenes* types 4a and 4d." *Journal of biochemistry* **97**: 883-891.

Gaillard, JL, P Berche, C Frehel, E Gouin and P Cossart (1991). "Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci." *Cell* **65**: 1127-1141.

Gaillard, JL, P Berche, J Mounier, S Richard and P Sansonetti (1987). "In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2." *Infect Immun* **55**: 2822-2829.

Gaillard, JL, P Berche and P Sansonetti (1986). "Transposon Mutagenesis as a Tool to Study the Role of Hemolysin in the Virulence of *Listeria Monocytogenes*." *Infect Immun* **52**(1): 50-55.

Gallo, RL, KJ Kim, M Bernfield, Ca Kozak, M Zanetti*, et al.* (1997). "Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse." *The Journal of biological chemistry* **272**: 13088-13093.

Gallo, RL, M Ono, T Povsic, C Page, E Eriksson*, et al.* (1994). "Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds." *Proceedings of the National Academy of Sciences of the United States of America* **91**(23): 11035-11039.

Galvez, A, M Maqueda, E Valdivia, A Quesada and E Montoya (1986). "Characterization and partial purification of a broad spectrum antibiotic AS-48

produced by *Streptococcus faecalis*." *Canadian journal of microbiology* **32**(10): 765-771.

Ganz, T (1987). "Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes." *Infect Immun* **55**(3): 568-571.

Ganz, T (2003). "Defensins: antimicrobial peptides of innate immunity." *Nature reviews Immunology* **3**: 710-720.

Ganz, T and RI Lehrer (1995). "Defensins." *Pharmacology & therapeutics* **66**(2): 191-205.

Ganz, T, ME Selsted, D Szklarek, SS Harwig, K Daher*, et al.* (1985). "Defensins. Natural peptide antibiotics of human neutrophils." *The Journal of clinical investigation* **76**(4): 1427-1435.

Gedde, MM, DE Higgins, LG Tilney and DA Portnoy (2000). "Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*." *Infect Immun* **68**(2): 999-1003.

Gennaro, R, B Skerlavaj and D Romeo (1989). "Purification, composition, and activity of two bactenecins, antibacterial peptides of bovine neutrophils." *Infect Immun* **57**(10): 3142-3146.

Gennaro, R and M Zanetti (2000). "Structural features and biological activities of the cathelicidin-derived antimicrobial peptides." *Biopolymers* **55**(1): 31-49.

Geoffroy, C, JL Gaillard, JE Alouf and P Berche (1987). "Purification, Characterization, and Toxicity of the Sulfhydryl-Activated Hemolysin Listeriolysin O from *Listeria Monocytogenes*." *Infect Immun* **55**(7): 1641-1646.

George, SM and BM Lund (1992). "The effect of culture medium and aeration on growth of *Listeria monocytogenes* at pH 4.5." *Letters in Applied Microbiology* **15**: 49-52.

Ghosh, D, E Porter, B Shen, SK Lee, D Wilk*, et al.* (2002). "Paneth cell trypsin is the processing enzyme for human defensin-5." *Nature immunology* **3**(6): 583-590.

Ghuysen, JM (1994). "Molecular structures of penicillin-binding proteins and beta-lactamases." *Trends in microbiology* **2**: 372-380.

Ginsberg, C, YH Zhang, YQ Yuan and S Walker (2006). "*In vitro* reconstitution of two essential steps in wall teichoic acid biosynthesis." *Acs Chem Biol* **1**(1): 25-28.

Giraud, MF and JH Naismith (2000). "The rhamnose pathway." *Current opinion in structural biology* **10**: 687-696.

Giuliani, A, G Pirri and SF Nicoletto (2007). "Antimicrobial peptides: an overview of a promising class of therapeutics." *Central European Journal of Biology* **2**: 1-33.

Glaser, P, L Frangeul, C Buchrieser, C Rusniok, A Amend*, et al.* (2001). "Comparative genomics of *Listeria* species." *Science (New York, NY)* **294**: 849-852.

Glomski, IJ, AL Decatur and DA Portnoy (2003). "*Listeria monocytogenes* mutants that fail to compartmentalize listerolysin O activity are cytotoxic, avirulent, and unable to evade host extracellular defenses." *Infect Immun* **71**(12): 6754-6765.

Gorski, L (2008). Phenotypic identification. *In*: Liu D. (ed.) *Handbook of Listeria monocytogenes*. CRC Press, 10.1201/9781420051414.ch5**:** 139-168.

Götz, F, S Perconti, P Popella, R Werner and M Schlag (2014). "Epidermin and gallidermin: Staphylococcal lantibiotics." *International journal of medical microbiology : IJMM* **304**: 63-71.

Gould, LH, Ka Walsh, AR Vieira, K Herman, IT Williams*, et al.* (2013). "Surveillance for foodborne disease outbreaks - United States, 1998-2008." *Morbidity and mortality weekly report Surveillance summaries (Washington, DC : 2002)* **62**: 1-34.

Goulet, V, C Hedberg, A Le Monnier and H De Valk (2008). "Increasing incidence of listeriosis in France and other European countries." *Emerging Infectious Diseases* **14**: 734-740.

Goulet, V, C Jacquet, P Martin, V Vaillant, E Laurent*, et al.* (2006). "Surveillance of human listeriosis in France, 2001-2003." *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* **11**(6): 79-81.

Graves, LM, LO Helsel, AG Steigerwalt, RE Morey, MI Daneshvar*, et al.* (2010). "*Listeria marthii* sp nov., isolated from the natural environment, Finger Lakes National Forest." *Int J Syst Evol Micr* **60**: 1280-1288.

Greiffenberg, L, W Goebel, KS Kim, I Weiglein, A Bubert*, et al.* (1998). "Interaction of *Listeria monocytogenes* with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells." *Infect Immun* **66**(11): 5260-5267.

Gründling, A, LS Burrack, HGA Bouwer and DE Higgins (2004). "*Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence." *Proceedings of the National Academy of Sciences of the United States of America* **101**: 12318-12323.

Guariglia-Oropeza, V and JD Helmann (2011). "*Bacillus subtilis* sigma(V) confers lysozyme resistance by activation of two cell wall modification pathways, peptidoglycan O-acetylation and D-alanylation of teichoic acids." *J Bacteriol* **193**(22): 6223-6232.

Gudmundsson, GH, KP Magnusson, BP Chowdhary, M Johansson, L Andersson*, et al.* (1995). "Structure of the gene for porcine peptide antibiotic PR-39, a cathelin gene family member: comparative mapping of the locus for the human

peptide antibiotic FALL-39." *Proceedings of the National Academy of Sciences of the United States of America* **92**(15): 7085-7089.

Guilhelmelli, F, N Vilela, P Albuquerque, LDS Derengowski, I Silva-Pereira*, et al.* (2013). "Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance." *Frontiers in microbiology* **4**: 353.

Guina, T, EC Yi, H Wang, M Hackett and SI Miller (2000). "A PhoP-regulated outer membrane protease of *Salmonella enterica* serovar typhimurium promotes resistance to alpha-helical antimicrobial peptides." *J Bacteriol* **182**(14): 4077-4086.

Guinane, CM, PD Cotter, RP Ross and C Hill (2006). "Contribution of penicillin-binding protein homologs to antibiotic resistance, cell morphology, and virulence of *Listeria monocytogenes* EGDe." *Antimicrob Agents Ch* **50**: 2824-2828.

Gunn, JS, SS Ryan, JC Van Velkinburgh, RK Ernst and SI Miller (2000). "Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar typhimurium." *Infect Immun* **68**(11): 6139-6146.

Guo, L, KB Lim, JS Gunn, B Bainbridge, RP Darveau*, et al.* (1997). "Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*." *Science* **276**(5310): 250-253.

Guo, L, KB Lim, CM Poduje, M Daniel, JS Gunn*, et al.* (1998). "Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides." *Cell* **95**(2): 189-198.

Gutkind, GO, SB Ogueta, AC de Urtiaga, ME Mollerach and RA de Torres (1990). "Participation of PBP 3 in the acquisition of dicloxacillin resistance in *Listeria monocytogenes*." *The Journal of antimicrobial chemotherapy* **25**: 751-758.

Guzman, CA, E Domann, M Rohde, D Bruder, A Darji*, et al.* (1996). "Apoptosis of mouse dendritic cells is triggered by listeriolysin, the major virulence determinant of *Listeria monocytogenes*." *Mol Microbiol* **20**(1): 119-126.

Haas, R, HU Koch and W Fischer (1984). "Alanyl Turnover from Lipoteichoic Acid to Teichoic Acid in *Staphylococcus Aureus*." *Fems Microbiol Lett* **21**(1): 27-31.

Hain, T, C Steinweg and T Chakraborty (2006a). "Comparative and functional genomics of *Listeria* spp." *Journal of biotechnology* **126**: 37-51.

Hain, T, C Steinweg, CT Kuenne, A Billion, R Ghai*, et al.* (2006b). "Whole-genome sequence of *Listeria welshimeri* reveals common steps in genome reduction with *Listeria innocua* as compared to *Listeria monocytogenes*." *J Bacteriol* **188**: 7405-7415.

Hallock, KJ, DK Lee and A Ramamoorthy (2003). "MSI-78, an analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain." *Biophys J* **84**(5): 3052-3060.

Halter, EL, K Neuhaus and S Scherer (2013). "*Listeria weihenstephanensis* sp nov., isolated from the water plant *Lemna trisulca* taken from a freshwater pond." *Int J Syst Evol Micr* **63**: 641-647.

Hammami, R, A Zouhir, C Le Lay, J Ben Hamida and I Fliss (2010). "BACTIBASE second release: a database and tool platform for bacteriocin characterization." *BMC microbiology* **10**: 22.

Hamon, MA and P Cossart (2011). "K+ Efflux Is Required for Histone H3 Dephosphorylation by *Listeria monocytogenes* Listeriolysin O and Other Pore-Forming Toxins." *Infect Immun* **79**(7): 2839-2846.

Hamon, MA, D Ribet, F Stavru and P Cossart (2012). "Listeriolysin O: the Swiss army knife of *Listeria*." *Trends in microbiology* **20**: 360-368.

Hancock, REW and DS Chapple (1999). Peptide antibiotics. Antimicrob Agents Ch. **43:** 1317-1323.

Hancock, REW and HG Sahl (2006). "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies." *Nat Biotechnol* **24**(12): 1551-1557.

Hankins, JV, JA Madsen, DK Giles, JS Brodbelt and MS Trent (2012). "Amino acid addition to *Vibrio cholerae* LPS establishes a link between surface remodeling in gram-positive and gram-negative bacteria." *Proceedings of the National Academy of Sciences of the United States of America* **109**(22): 8722-8727.

Hara, T, H Kodama, M Kondo, K Wakamatsu, A Takeda*, et al.* (2001). "Effects of peptide dimerization on pore formation: Antiparallel disulfide-dimerized magainin 2 analogue." *Biopolymers* **58**(4): 437-446.

Harris, F, SR Dennison and DA Phoenix (2009). "Anionic Antimicrobial Peptides from Eukaryotic Organisms." *Curr Protein Pept Sc* **10**(6): 585-606.

Harwig, SS, AS Park and RI Lehrer (1992). "Characterization of defensin precursors in mature human neutrophils." *Blood* **79**(6): 1532-1537.

Hauge, HH, J Nissen-Meyer, IF Nes and VG Eijsink (1998). "Amphiphilic alpha-helices are important structural motifs in the alpha and beta peptides that constitute the bacteriocin lactococcin G--enhancement of helix formation upon alpha-beta interaction." *Eur J Biochem* **251**(3): 565-572.

Hayashi, K (1975). "A rapid determination of sodium dodecyl sulfate with methylene blue." *Analytical biochemistry* **67**: 503-506.

Hazlett, L and M Wu (2011). "Defensins in innate immunity." *Cell and tissue research* **343**: 175-188.

Hechard, Y, C Pelletier, Y Cenatiempo and J Frere (2001). "Analysis of sigma(54)-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EII(Man)) is involved in sensitivity to a bacteriocin, mesentericin Y105." *Microbiology* **147**(Pt 6): 1575-1580.

Heilmann, C, M Hussain, G Peters and F Götz (1997). "Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface." *Mol Microbiol* **24**: 1013-1024.

Hell, W, HG Meyer and SG Gatermann (1998). "Cloning of aas, a gene encoding a *Staphylococcus saprophyticus* surface protein with adhesive and autolytic properties." *Mol Microbiol* **29**: 871-881.

Henderson, JT, AL Chopko and PD van Wassenaar (1992). "Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0." *Archives of biochemistry and biophysics* **295**(1): 5-12.

Hendrickx, APa, RJL Willems, MJM Bonten and W van Schaik (2009). "LPxTG surface proteins of enterococci." *Trends in microbiology* **17**: 423-430.

Henzler Wildman, KA, DK Lee and A Ramamoorthy (2003). "Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37." *Biochemistry* **42**(21): 6545-6558.

Hernandez-Milian, A and A Payeras-Cifre (2014). "What is new in Listeriosis?" *BioMed Research International* **2014**.

Hertz, CJ, Q Wu, EM Porter, YJ Zhang, KH Weismuller*, et al.* (2003). "Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2." *J Immunol* **171**(12): 6820-6826.

Hess, J, A Dreher, I Gentschev, W Goebel, C Ladel*, et al.* (1996). "Protein p60 participates in intestinal host invasion by *Listeria monocytogenes*." *Zentralblatt für Bakteriologie : international journal of medical microbiology* **284**: 263-272.

Hether, NW and LL Jackson (1983). "Lipoteichoic acid from *Listeria monocytogenes*." *J Bacteriol* **156**: 809-817.

Holo, H, O Nilssen and IF Nes (1991). "Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. cremoris: isolation and characterization of the protein and its gene." *J Bacteriol* **173**(12): 3879-3887.

Holtje, JV and A Tomasz (1975). "Specific recognition of choline residues in the cell wall teichoic acid by the N-acetylmuramyl-L-alanine amidase of *Pneumococcus*." *J Biol Chem* **250**(15): 6072-6076.

Hsu, STD, E Breukink, E Tischenko, MAG Lutters, B de Kruijff*, et al.* (2004). "The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics." *Nat Struct Mol Biol* **11**(10): 963-967.

Huang, HJ, CR Ross and F Blecha (1997). "Chemoattractant properties of PR-39, a neutrophil antibacterial peptide." *Journal of leukocyte biology* **61**(5): 624-629.

Huang, LC, RY Reins, RL Gallo and AM McDermott (2007). "Cathelicidin-deficient (Cnlp -/-) mice show increased susceptibility to *Pseudomonas aeruginosa* keratitis." *Investigative ophthalmology & visual science* **48**: 4498-4508.

Hurst, A, A Hughes, M Duckworth and J Baddiley (1975). "Loss of D-Alanine during Sublethal Heating of *Staphylococcus Aureus* S6 and Magnesium Binding during Repair." *J Gen Microbiol* **89**(Aug): 277-284.

Hutchings, MI, T Palmer, DJ Harrington and IC Sutcliffe (2009). "Lipoprotein biogenesis in Gram-positive bacteria: knowing when to hold 'em, knowing when to fold 'em." *Trends in microbiology* **17**: 13-21.

Ireton, K, B Payrastre, H Chap, W Ogawa, H Sakaue*, et al.* (1996). "A role for phosphoinositide 3-kinase in bacterial invasion." *Science* **274**(5288): 780-782.

Islam, MR, Ji Nagao, T Zendo and K Sonomoto (2012). "Antimicrobial mechanism of lantibiotics." *Biochemical Society Transactions* **40**: 1528-1533.

Iwasaki, H, A Shimada and E Ito (1986). "Comparative Studies of Lipoteichoic Acids from Several *Bacillus* Strains." *J Bacteriol* **167**(2): 508-516.

Iwasaki, H, A Shimada, K Yokoyama and E Ito (1989). "Structure and glycosylation of lipoteichoic acids in *Bacillus strains*." *J Bacteriol* **171**: 424-429.

Jacquet, C, E Gouin, D Jeannel, P Cossart and J Rocourt (2002). "Expression of ActA, Ami, InlB, and listeriolysin O in *Listeria monocytogenes* of human and food origin." *Applied and environmental microbiology* **68**(2): 616-622.

Jin, T, M Bokarewa, T Foster, J Mitchell, J Higgins*, et al.* (2004). "*Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism." *J Immunol* **172**(2): 1169-1176.

Johnsen, L, G Fimland and JN Meyer (2005). "The C-terminal domain of pediocin-like antimicrobial peptides (class IIa bacteriocins) is involved in specific recognition of the C-terminal part of cognate immunity proteins and in determining the antimicrobial spectrum." *J Biol Chem* **280**(10): 9243-9250.

Jonquières, R, H Bierne, F Fiedler, P Gounon and P Cossart (1999). "Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of gram-positive bacteria." *Mol Microbiol* **34**: 902-914.

Jonquières, R, J Pizarro-Cerdá and P Cossart (2001). "Synergy between the N- and C-terminal domains of InlB for efficient invasion of non-phagocytic cells by *Listeria monocytogenes*." *Mol Microbiol* **42**: 955-965.

Jorasch, P, DC Warnecke, B Lindner, U Zahringer and E Heinz (2000). "Novel processive and nonprocessive glycosyltransferases from *Staphylococcus*

aureus and *Arabidopsis thaliana* synthesize glycoglycerolipids, glycophospholipids, glycosphingolipids and glycosylsterols." *Eur J Biochem* **267**(12): 3770-3783.

Joris, B, S Englebert, CP Chu, R Kariyama, L Daneo-Moore*, et al.* (1992). "Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin." *Fems Microbiol Lett* **70**: 257-264.

Joseph, B, K Przybilla, C Stühler, K Schauer, J Slaghuis*, et al.* (2006). "Identification of *Listeria monocytogenes* genes contributing to intracellular replication by expression profiling and mutant screening." *J Bacteriol* **188**: 556-568.

Juergens, WG, AR Sanderson and JL Strominger (1963). "Chemical basis for an immunological specificity of a strain of *Staphylococcus aureus*." *The Journal of experimental medicine* **117**: 925-935.

Jung, G (1991). Lantibiotics: a survey. *In*: Jung G. and H. G. Sahl (eds.), *Nisin and novel lantibiotics*. Leiden, Escom**:** 1-34.

Junttila, JR, SI Niemela and J Hirn (1988). "Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*." *The Journal of applied bacteriology* **65**(4): 321-327.

Kai-Larsen, Y and B Agerberth (2008). "The role of the multifunctional peptide LL-37 in host defense." *Frontiers in bioscience : a journal and virtual library* **13**: 3760-3767.

Kaiser, V and G Diamond (2000). "Expression of mammalian defensin genes." *Journal of leukocyte biology* **68**: 779-784.

Kamisango, K, I Saiki, Y Tanio, H Okumura, Y Araki*, et al.* (1982). "Structures and biological activities of peptidoglycans of *Listeria monocytogenes* and *Propionibacterium acnes*." *Journal of biochemistry* **92**(1): 23-33.

Kamisango, K-i, H Fujii, H Okumura, I Saiki, Y Araki*, et al.* (1983). "Structural and immunochemical studies of teichoic acid of *Listeria monocytogenes*." *Journal of biochemistry* **93**: 1401-1409.

Kaneko, T, L Li and SS Li (2008). "The SH3 domain--a family of versatile peptide- and protein-recognition module." *Frontiers in bioscience : a journal and virtual library* **13**: 4938-4952.

Kathariou, S, P Metz, H Hof and W Goebel (1987). "Tn916-Induced Mutations in the Hemolysin Determinant Affecting Virulence of *Listeria Monocytogenes*." *J Bacteriol* **169**(3): 1291-1297.

Kawulka, K, T Sprules, RT McKay, P Mercier, CM Diaper*, et al.* (2003). "Structure of subtilosin A, an antimicrobial peptide from *Bacillus subtilis* with unusual posttranslational modifications linking cysteine sulfurs to alpha-carbons of phenylalanine and threonine." *J Am Chem Soc* **125**(16): 4726-4727.

Kaya, S, Y Araki and E Ito (1985). "Characterization of a novel linkage unit between ribitol teichoic acid and peptidoglycan in *Listeria monocytogenes* cell walls." *European journal of biochemistry / FEBS* **146**: 517-522.

Kayal, S, A Lilienbaum, C Poyart, S Memet, A Israel*, et al.* (1999). "Listeriolysin O-dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-kappa B and upregulation of adhesion molecules and chemokines." *Mol Microbiol* **31**(6): 1709-1722.

Kellner, R, G Jung, T Hörner, H Zähner, N Schnell*, et al.* (1988). "Gallidermin: a new lanthionine-containing polypeptide antibiotic." *European journal of biochemistry / FEBS* **177**: 53-59.

Kern, T, M Giffard, S Hediger, A Amoroso, C Giustini*, et al.* (2010). "Dynamics Characterization of Fully Hydrated Bacterial Cell Walls by Solid-State NMR: Evidence for Cooperative Binding of Metal Ions." *J Am Chem Soc* **132**(31): 10911-10919.

Kirchner, M and DE Higgins (2008). "Inhibition of ROCK activity allows InlF-mediated invasion and increased virulence of *Listeria monocytogenes*." *Mol Microbiol* **68**: 749-767.

Kirikae, T, M Hirata, H Yamasu, F Kirikae, H Tamura*, et al.* (1998). "Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia." *Infect Immun* **66**(5): 1861-1868.

Kiriukhin, MY, DV Debabov, DL Shinabarger and FC Neuhaus (2001). "Biosynthesis of the glycolipid anchor in lipoteichoic acid of *Staphylococcus aureus* RN4220: Role of YpfP, the diglucosyldiacylglycerol synthase." *J Bacteriol* **183**(11): 3506-3514.

Kjos, M, C Oppegård, DB Diep, IF Nes, J-W Veening*, et al.* (2014). "Sensitivity to the two-peptide bacteriocin lactococcin G is dependent on UppP, an enzyme involved in cell-wall synthesis." *Mol Microbiol* **92**: 1177-1187.

Klebba, PE, A Charbit, Q Xiao, X Jiang and SM Newton (2012). "Mechanisms of iron and haem transport by *Listeria monocytogenes*." *Molecular membrane biology* **29**: 69-86.

Kluver, E, S Schulz-Maronde, S Scheid, B Meyer, WG Forssmann*, et al.* (2005). "Structure-activity relation of human beta-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity." *Biochemistry* **44**(28): 9804-9816.

Kocks, C, E Gouin, M Tabouret, P Berche, H Ohayon*, et al.* (1992). "*L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein." *Cell* **68**: 521-531.

Kocks, C, JB Marchand, E Gouin, H D'Hauteville, PJ Sansonetti*, et al.* (1995). "The unrelated surface proteins ActA of *Listeria monocytogenes* and IcsA of

Shigella flexneri are sufficient to confer actin-based motility on *Listeria innocua* and *Escherichia coli* respectively." *Mol Microbiol* **18**: 413-423.

Kohler, T, C Weidenmaier and A Peschel (2009). "Wall Teichoic Acid Protects *Staphylococcus aureus* against Antimicrobial Fatty Acids from Human Skin." *J Bacteriol* **191**(13): 4482-4484.

Kooi, C and PA Sokol (2009). "*Burkholderia cenocepacia* zinc metalloproteases influence resistance to antimicrobial peptides." *Microbiology* **155**(Pt 9): 2818-2825.

Koprivnjak, T and A Peschel (2011). "Bacterial resistance mechanisms against host defense peptides." *Cellular and molecular life sciences : CMLS* **68**: 2243-2254.

Koprivnjak, T, A Peschel, MH Gelb, NS Liang and JP Weiss (2002). "Role of charge properties of bacterial envelope in bactericidal action of human group IIA phospholipase A2 against *Staphylococcus aureus*." *The Journal of biological chemistry* **277**: 47636-47644.

Korsak, D, Z Markiewicz, GO Gutkind and Ja Ayala (2010). "Identification of the full set of *Listeria monocytogenes* penicillin-binding proteins and characterization of PBPD2 (Lmo2812)." *BMC microbiology* **10**: 239.

Kościuczuk, EM, P Lisowski, J Jarczak, N Strzałkowska, A Jóźwik*, et al.* (2012). "Cathelicidins: family of antimicrobial peptides. A review." *Molecular Biology Reports*, 10.1007/s11033-012-1997-x: 1-14.

Köster, S, K van Pee, M Hudel, M Leustik, D Rhinow*, et al.* (2014). "Crystal structure of listeriolysin O reveals molecular details of oligomerization and pore formation." *Nature communications* **5**: 3690.

Kovacs, M, A Halfmann, I Fedtke, M Heintz, A Peschel*, et al.* (2006). "A Functional *dlt* Operon, Encoding Proteins Required for Incorporation of D-Alanine in Teichoic Acids in Gram-Positive Bacteria, Confers Resistance to Cationic Antimicrobial Peptides in *Streptococcus pneumoniae*." *J Bacteriol* **188**: 5797-5805.

Kovacs-Simon, a, RW Titball and SL Michell (2011). "Lipoproteins of bacterial pathogens." *Infect Immun* **79**: 548-561.

Kragol, G, S Lovas, G Varadi, BA Condie, R Hoffmann*, et al.* (2001). "The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding." *Biochemistry* **40**(10): 3016-3026.

Kristian, SA, V Datta, C Weidenmaier, R Kansal, I Fedtke*, et al.* (2005). "D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion." *J Bacteriol* **187**(19): 6719-6725.

Kristian, SA, M Durr, JA Van Strijp, B Neumeister and A Peschel (2003). "MprF-mediated lysinylation of phospholipids in *Staphylococcus aureus* leads to protection against oxygen-independent neutrophil killing." *Infect Immun* **71**(1): 546-549.

Kuenne, C, A Billion, M Abu Mraheil, A Strittmatter, R Daniel*, et al.* (2013). "Reassessment of the *Listeria monocytogenes* pan-genome reveals dynamic integration hotspots and mobile genetic elements as major components of the accessory genome." *Bmc Genomics* **14**.

Kuhn, M and W Goebel (1989). "Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells." *Infect Immun* **57**: 55-61.

La, MV, D Raoult and P Renesto (2008). "Regulation of whole bacterial pathogen transcription within infected hosts." *Fems Microbiol Rev* **32**: 440-460.

Ladokhin, AS and SH White (2001). "'Detergent-like' permeabilization of anionic lipid vesicles by melittin." *Biochim Biophys Acta* **1514**(2): 253-260.

Lairson, LL, B Henrissat, GJ Davies and SG Withers (2008). "Glycosyltransferases: structures, functions, and mechanisms." *Annu Rev Biochem* **77**: 521-555.

Lamont, RF, J Sobel, S Mazaki-Tovi, JP Kusanovic, E Vaisbuch*, et al.* (2011). "Listeriosis in human pregnancy: A systematic review." *Journal of Perinatal Medicine* **39**: 227-236.

Larrick, JW, M Hirata, RF Balint, J Lee, J Zhong*, et al.* (1995). "Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein." *Infect Immun* **63**(4): 1291-1297.

Larrick, JW, M Hirata, H Zheng, J Zhong, D Bolin*, et al.* (1994). "A novel granulocyte-derived peptide with lipopolysaccharide-neutralizing activity." *J Immunol* **152**(1): 231-240.

Larrick, JW, J Lee, S Ma, X Li, U Francke*, et al.* (1996). "Structural, functional analysis and localization of the human CAP18 gene." *Febs Lett* **398**(1): 74-80.

Larsen, HE and HP Seeliger (1966). "A mannitol fermenting *Listeria*, *Listeria grayi* sp. n." *Proceedings of the Third International Symposium on Listeriosis*: 35.

Lasa, I, V David, E Gouin, JB Marchand and P Cossart (1995). "The amino-terminal part of ActA is critical for the actin-based motility of *Listeria monocytogenes*; the central proline-rich region acts as a stimulator." *Mol Microbiol* **18**: 425-436.

Lasa, I, E Gouin, M Goethals, K Vancompernolle, V David*, et al.* (1997). "Identification of two regions in the N-terminal domain of ActA involved in the actin comet tail formation by *Listeria monocytogenes*." *Embo J* **16**: 1531-1540.

Lauer, P, MYN Chow, MJ Loessner, DA Portnoy and R Calendar (2002). "Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors." *J Bacteriol* **184**: 4177-4186.

Lauth, X, M von Kockritz-Blickwede, CW McNamara, S Myskowski, AS Zinkernagel*, et al.* (2009). "M1 protein allows Group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition." *Journal of innate immunity* **1**(3): 202-214.

Lawton, EM, RP Ross, C Hill and PD Cotter (2007). "Two-peptide lantibiotics: a medical perspective." *Mini reviews in medicinal chemistry* **7**(12): 1236-1247.

Layec, S, B Decaris and N Leblond-Bourget (2008). "Characterization of proteins belonging to the CHAP-related superfamily within the Firmicutes." *Journal of molecular microbiology and biotechnology* **14**: 31-40.

Leclercq, A, D Clermont, C Bizet, PAD Grimont, A Le Fleche-Mateos*, et al.* (2010). "*Listeria rocourtiae* sp. nov." *Int J Syst Evol Micr* **60**: 2210-2214.

Lecuit, M (2005). "Understanding how *Listeria monocytogenes* targets and crosses host barriers." *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **11**: 430-436.

Lecuit, M (2007). "Human listeriosis and animal models." *Microbes and infection / Institut Pasteur* **9**: 1216-1225.

Lecuit, M, S Dramsi, C Gottardi, M Fedor-Chaiken, B Gumbiner*, et al.* (1999). "A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*." *Embo J* **18**(14): 3956-3963.

Lecuit, M, DM Nelson, SD Smith, H Khun, M Huerre*, et al.* (2004). "Targeting and crossing of the human maternofetal barrier by *Listeria monocytogenes*: role of internalin interaction with trophoblast E-cadherin." *Proceedings of the National Academy of Sciences of the United States of America* **101**(16): 6152-6157.

Lee, JY, ST Yang, HJ Kim, SK Lee, HH Jung*, et al.* (2009). "Different modes of antibiotic action of homodimeric and monomeric bactenecin, a cathelicidin-derived antibacterial peptide." *BMB reports* **42**(9): 586-592.

Lee, MT, FY Chen and HW Huang (2004). "Energetics of pore formation induced by membrane active peptides." *Biochemistry* **43**(12): 3590-3599.

Lenz, LL, S Mohammadi, A Geissler and Da Portnoy (2003). "SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis." *Proceedings of the National Academy of Sciences of the United States of America* **100**: 12432-12437.

Lety, MA, C Frehel, I Dubail, JL Beretti, S Kayal*, et al.* (2001). "Identification of a PEST-like motif in listeriolysin O required for phagosomal escape and for virulence in *Listeria monocytogenes*." *Mol Microbiol* **39**: 1124-1139.

Li, Q, M Hobbs and PR Reeves (2003). "The variation of dTDP-L-rhamnose pathway genes in *Vibrio cholerae*." *Microbiology (Reading, England)* **149**: 2463-2474.

Li, Q and PR Reeves (2000). "Genetic variation of dTDP-L-rhamnose pathway genes in *Salmonella enterica*." *Microbiology* **146 (Pt 9**: 2291-2307.

Li, W, Y Xin, MR McNeil and Y Ma (2006). "*rmlB* and *rmlC* genes are essential for growth of mycobacteria." *Biochemical and biophysical research communications* **342**: 170-178.

Lingnau, A, E Domann, M Hudel, M Bock, T Nichterlein*, et al.* (1995). "Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms." *Infect Immun* **63**: 3896-3903.

Liu, D, ML Lawrence, aJ Ainsworth and FW Austin (2007). "Toward an improved laboratory definition of *Listeria monocytogenes* virulence." *Int J Food Microbiol* **118**: 101-115.

Liu, L, AA Roberts and T Ganz (2003). "By IL-1 signaling, monocyte-derived cells dramatically enhance the epidermal antimicrobial response to lipopolysaccharide." *J Immunol* **170**(1): 575-580.

Llobet, E, JM Tomas and JA Bengoechea (2008). "Capsule polysaccharide is a bacterial decoy for antimicrobial peptides." *Microbiology* **154**(Pt 12): 3877-3886.

Lohner, K, A Latal, G Degovics and P Garidel (2001). "Packing characteristics of a model system mimicking cytoplasmic bacterial membranes." *Chem Phys Lipids* **111**(2): 177-192.

Lucero, CM, B Fallert Junecko, CR Klamar, LA Sciullo, SJ Berendam*, et al.* (2013). "Macaque paneth cells express lymphoid chemokine CXCL13 and other antimicrobial peptides not previously described as expressed in intestinal crypts." *Clin Vaccine Immunol* **20**(8): 1320-1328.

Lynch, NJ, S Roscher, T Hartung, S Morath, M Matsushita*, et al.* (2004). "L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of gram-positive bacteria, and activates the lectin pathway of complement." *J Immunol* **172**(2): 1198-1202.

Ma, Y, RJ Stern, MS Scherman, VD Vissa, W Yan*, et al.* (2001). "Drug targeting *Mycobacterium tuberculosis* cell wall synthesis: genetics of dTDP-rhamnose synthetic enzymes and development of a microtiter plate-based screen for inhibitors of conversion of dTDP-glucose to dTDP-rhamnose." *Antimicrob Agents Chemother* **45**(5): 1407-1416.

Macarthur, AE and AR Archibald (1984). "Effect of Culture pH on the D-Alanine Ester Content of Lipoteichoic Acid in *Staphylococcus Aureus*." *J Bacteriol* **160**(2): 792-793.

Macheboeuf, P, C Contreras-Martel, V Job, O Dideberg and A Dessen (2006). "Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes." *Fems Microbiol Rev* **30**: 673-691.

Mackaness, GB (1960). "The phagocytosis and inactivation of staphylococci by macrophages of normal rabbits." *The Journal of experimental medicine* **112**: 35-53.

Macpherson, DF, PA Manning and R Morona (1994). "Characterization of the dTDP-rhamnose biosynthetic genes encoded in the *rfb* locus of *Shigella flexneri*." *Mol Microbiol* **11**: 281-292.

Mandin, P, H Fsihi, O Dussurget, M Vergassola, E Milohanic*, et al.* (2005). "VirR, a response regulator critical for *Listeria monocytogenes* virulence." *Mol Microbiol* **57**: 1367-1380.

Maqueda, M, M Sánchez-Hidalgo, M Fernández, M Montalbán-López, E Valdivia*, et al.* (2008). "Genetic features of circular bacteriocins produced by Gram-positive bacteria." *Fems Microbiol Rev* **32**: 2-22.

Maresso, aW and O Schneewind (2006). "Iron acquisition and transport in *Staphylococcus aureus*." *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* **19**: 193-203.

Marino, M, M Banerjee, R Jonquières, P Cossart and P Ghosh (2002). "GW domains of the *Listeria monocytogenes* invasion protein InlB are SH3-like and mediate binding to host ligands." *The EMBO journal* **21**: 5623-5634.

Mariscotti, JF, F García-del Portillo and MG Pucciarelli (2009). "The *Listeria monocytogenes* sortase B recognizes varied amino acids at position 2 of the sorting motif." *The Journal of biological chemistry* **284**: 6140-6146.

Marquis, H, V Doshi and DA Portnoy (1995). "The Broad-Range Phospholipase-C and a Metalloprotease Mediate Listeriolysin O-Independent Escape of *Listeria Monocytogenes* from a Primary Vacuole in Human Epithelial Cells." *Infect Immun* **63**(11): 4531-4534.

Marquis, RE, K Mayzel and EL Carstensen (1976). "Cation exchange in cell walls of gram-positive bacteria." *Canadian journal of microbiology* **22**: 975-982.

Marraffini, LA, AC Dedent and O Schneewind (2006). "Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria." *Microbiology and molecular biology reviews : MMBR* **70**: 192-221.

Martin, NI, T Sprules, MR Carpenter, PD Cotter, C Hill*, et al.* (2004). "Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity." *Biochemistry* **43**(11): 3049-3056.

Martinez-Bueno, M, M Maqueda, A Galvez, B Samyn, J Van Beeumen*, et al.* (1994). "Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48." *J Bacteriol* **176**(20): 6334-6339.

Matsuzaki, K, O Murase, N Fujii and K Miyajima (1996). "An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation." *Biochemistry* **35**(35): 11361-11368.

Mazmanian, SK, G Liu, ER Jensen, E Lenoy and O Schneewind (2000). "*Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections." *Proceedings of the National Academy of Sciences of the United States of America* **97**: 5510-5515.

Mazmanian, SK, H Ton-That and O Schneewind (2001). "Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*." *Mol Microbiol* **40**: 1049-1057.

Mazmanian, SK, H Ton-That, K Su and O Schneewind (2002). "An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis." *Proceedings of the National Academy of Sciences of the United States of America* **99**: 2293-2298.

McAuliffe, O, MP Ryan, RP Ross, C Hill, P Breeuwer*, et al.* (1998). "Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential." *Applied and environmental microbiology* **64**(2): 439-445.

McBride, SM and AL Sonenshein (2011). "The *dlt* operon confers resistance to cationic antimicrobial peptides in *Clostridium difficile*." *Microbiology* **157**(Pt 5): 1457-1465.

McLaughlan, aM and SJ Foster (1998). "Molecular characterization of an autolytic amidase of *Listeria monocytogenes* EGD." *Microbiology (Reading, England)* **144 (Pt 5**: 1359-1367.

Ménard, S, V Förster, M Lotz, D Gütle, CU Duerr*, et al.* (2008). "Developmental switch of intestinal antimicrobial peptide expression." *The Journal of experimental medicine* **205**: 183-193.

Mengaud, J, H Ohayon, P Gounon, RM Mege and P Cossart (1996). "E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells." *Cell* **84**(6): 923-932.

Michaelson, D, J Rayner, M Couto and T Ganz (1992). "Cationic defensins arise from charge-neutralized propeptides: a mechanism for avoiding leukocyte autocytotoxicity?" *Journal of leukocyte biology* **51**(6): 634-639.

Mihajlovic, M and T Lazaridis (2010). "Antimicrobial peptides bind more strongly to membrane pores." *Bba-Biomembranes* **1798**(8): 1494-1502.

Miles, K, DJ Clarke, W Lu, Z Sibinska, PE Beaumont*, et al.* (2009). "Dying and necrotic neutrophils are anti-inflammatory secondary to the release of alpha-defensins." *J Immunol* **183**(3): 2122-2132.

Milohanic, E, P Glaser, J-Y Coppée, L Frangeul, Y Vega*, et al.* (2003). "Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA." *Mol Microbiol* **47**: 1613-1625.

Milohanic, E, R Jonquières, P Cossart, P Berche and J-LL Gaillard (2001). "The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor." *Mol Microbiol* **39**: 1212-1224.

Milohanic, E, R Jonquières, P Glaser, P Dehoux, C Jacquet*, et al.* (2004). "Sequence and binding activity of the autolysin-adhesin Ami from epidemic Listeria monocytogenes 4b." *Infect Immun* **72**: 4401-4409.

Milohanic, E, B Pron, P Berche and JL Gaillard (2000). "Identification of new loci involved in adhesion of *Listeria monocytogenes* to eukaryotic cells. European *Listeria* Genome Consortium." *Microbiology (Reading, England)* **146 (Pt 3**: 731-739.

Moll, G, T Ubbink-Kok, H Hildeng-Hauge, J Nissen-Meyer, IF Nes*, et al.* (1996). "Lactococcin G is a potassium ion-conducting, two-component bacteriocin." *J Bacteriol* **178**(3): 600-605.

Morath, S, A Geyer and T Hartung (2001). "Structure-function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*." *Journal of Experimental Medicine* **193**(3): 393-397.

Morin, N, I Lanneluc, N Connil, M Cottenceau, AM Pons*, et al.* (2011). "Mechanism of bactericidal activity of microcin L in *Escherichia coli* and *Salmonella enterica*." *Antimicrob Agents Chemother* **55**(3): 997-1007.

Munk, C, G Wei, OO Yang, AJ Waring, W Wang*, et al.* (2003). "The theta-defensin, retrocyclin, inhibits HIV-1 entry." *AIDS research and human retroviruses* **19**(10): 875-881.

Murakami, M, B Lopez-Garcia, M Braff, RA Dorschner and RL Gallo (2004). "Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense." *J Immunol* **172**(5): 3070-3077.

Murray, EGD, RA Webb and MBR Swann (1926). "A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes*." *The Journal of Pathology and Bacteriology* **29**: 407-439.

Nagaoka, I, F Niyonsaba, Y Tsutsumi-Ishii, H Tamura and M Hirata (2008). "Evaluation of the effect of human beta-defensins on neutrophil apoptosis." *International immunology* **20**(4): 543-553.

Nakao, A, S Imai and T Takano (2000). "Transposon-mediated insertional mutagenesis of the D-alanyl-lipoteichoic acid (*dlt*) operon raises methicillin resistance in *Staphylococcus aureus*." *Research in microbiology* **151**(10): 823-829.

Nakayama, H, K Kurokawa and BL Lee (2012). "Lipoproteins in bacteria: structures and biosynthetic pathways." *The FEBS journal* **279**: 4247-4268.

Nathenson, SG, N Ishimoto, JS Anderson and JL Strominger (1966). "Enzymatic synthesis and immunochemistry of alpha- and beta-*N*-acetylglucosaminylribitol linkages in teichoic acids from several strains of *Staphylococcus aureus*." *J Biol Chem* **241**(3): 651-658.

Naumova, IB, AS Shashkov, EM Tul'Skaya, GM Streshinskaya, YI Kozlova*, et al.* (2001). "Cell wall teichoic acids: Structural diversity, species specificity in the genus *Nocardiopsis*, and chemotaxonomic perspective." *Fems Microbiol Rev* **25**: 269-283.

Navarre, WW and O Schneewind (1999). "Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope." *Microbiology and molecular biology reviews : MMBR* **63**: 174-229.

Nelson, KE, DE Fouts, EF Mongodin, J Ravel, RT DeBoy*, et al.* (2004). "Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species." *Nucleic Acids Res* **32**: 2386-2395.

Neuhaus, FC and J Baddiley (2003). "A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria." *Microbiology and molecular biology reviews : MMBR* **67**: 686-723.

Newton, SMC, PE Klebba, C Raynaud, Y Shao, X Jiang*, et al.* (2005). "The *svpA-srtB* locus of *Listeria monocytogenes*: fur-mediated iron regulation and effect on virulence." *Mol Microbiol* **55**: 927-940.

Nguyen, LT, EF Haney and HJ Vogel (2011). "The expanding scope of antimicrobial peptide structures and their modes of action." *Trends in biotechnology* **29**: 464-472.

Nguyen, TX, AM Cole and RI Lehrer (2003). "Evolution of primate theta-defensins: a serpentine path to a sweet tooth." *Peptides* **24**(11): 1647-1654.

Nilsen, T, IF Nes and H Holo (2003). "Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333." *Applied and environmental microbiology* **69**(5): 2975-2984.

Nishibori, T, HB Xiong, I Kawamura, M Arakawa and M Mitsuyama (1996). "Induction of cytokine gene expression by listeriolysin O and roles of macrophages and NK cells." *Infect Immun* **64**(8): 3188-3195.

Nissen-Meyer, J, H Holo, LS Havarstein, K Sletten and IF Nes (1992). "A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides." *J Bacteriol* **174**(17): 5686-5692.

Nissen-Meyer, J, P Rogne, C Oppegård, HS Haugen and PE Kristiansen (2009). "Structure-function relationships of the non-lanthionine-containing peptide

(class II) bacteriocins produced by gram-positive bacteria." *Current pharmaceutical biotechnology* **10**: 19-37.

Nizet, V, T Ohtake, X Lauth, J Trowbridge, J Rudisill*, et al.* (2001). "Innate antimicrobial peptide protects the skin from invasive bacterial infection." *Nature* **414**: 454-457.

Novak, R, JS Braun, E Charpentier and E Tuomanen (1998). "Penicillin tolerance genes of *Streptococcus pneumoniae*: the ABC-type manganese permease complex Psa." *Mol Microbiol* **29**: 1285-1296.

Novo, D, NG Perlmutter, RH Hunt and HM Shapiro (1999). "Accurate flow cytometric membrane potential measurement in bacteria using diethyloxacarbocyanine and a ratiometric technique." *Cytometry* **35**: 55-63.

Nyberg, P, M Rasmussen and L Bjorck (2004). "alpha2-Macroglobulin-proteinase complexes protect *Streptococcus pyogenes* from killing by the antimicrobial peptide LL-37." *J Biol Chem* **279**(51): 52820-52823.

Nyfeldt, A (1929). "Etiologie de la mononucléose infectieuse." *Comptes Rendus de la Societé de Biologie* **101**: 590-592.

Oppegard, C, G Fimland, L Thorbaek and J Nissen-Meyer (2007). "Analysis of the two-peptide bacteriocins lactococcin G and enterocin 1071 by site-directed mutagenesis." *Applied and environmental microbiology* **73**(9): 2931-2938.

Oren, Z and Y Shai (1998). "Mode of action of linear amphipathic alpha-helical antimicrobial peptides." *Biopolymers* **47**(6): 451-463.

Ornelas-Soares, A, H De Lencastre, BLM De Jonge and A Tomasz (1994). "Reduced methicillin resistance in a new *Staphylococcus aureus* transposon mutant that incorporates muramyl dipeptides into the cell wall peptidoglycan." *J Biol Chem* **269**: 27246-27250.

Orsi, RH, HC den Bakker and M Wiedmann (2011). "*Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics." *Int J Med Microbiol* **301**(2): 79-96.

Oshida, T, M Sugai, H Komatsuzawa, YM Hong, H Suginaka*, et al.* (1995). "A *Staphylococcus aureus* autolysin that has an N-acetylmuramoyl-L-alanine amidase domain and an endo-beta-N-acetylglucosaminidase domain: cloning, sequence analysis, and characterization." *Proceedings of the National Academy of Sciences of the United States of America* **92**: 285-289.

Otvos, L, Jr., I O, ME Rogers, PJ Consolvo, BA Condie*, et al.* (2000). "Interaction between heat shock proteins and antimicrobial peptides." *Biochemistry* **39**(46): 14150-14159.

Ouellette, AJ, RM Greco, M James, D Frederick, J Naftilan*, et al.* (1989). "Developmental regulation of cryptdin, a corticostatin/defensin precursor

mRNA in mouse small intestinal crypt epithelium." *The Journal of cell biology* **108**(5): 1687-1695.

Paetzel, M, RE Dalbey and NC Strynadka (2000). "The structure and mechanism of bacterial type I signal peptidases. A novel antibiotic target." *Pharmacology & therapeutics* **87**: 27-49.

Palumbo, E, M Deghorain, PS Cocconcelli, M Kleerebezem, A Geyer*, et al.* (2006). "D-alanyl ester depletion of teichoic acids in *Lactobacillus plantarum* results in a major modification of lipoteichoic acid composition and cell wall perforations at the septum mediated by the Acm2 autolysin." *J Bacteriol* **188**(10): 3709-3715.

Panyutich, A, J Shi, PL Boutz, C Zhao and T Ganz (1997). "Porcine polymorphonuclear leukocytes generate extracellular microbicidal activity by elastase-mediated activation of secreted proprotegrins." *Infect Immun* **65**(3): 978-985.

Parida, SK, E Domann, M Rohde, S Müller, A Darji*, et al.* (1998). "Internalin B is essential for adhesion and mediates the invasion of *Listeria monocytogenes* into human endothelial cells." *Mol Microbiol* **28**: 81-93.

Parish, ME and DE Higgins (1989). "Survival of *Listeria monocytogenes* in low pH model broth systems." *Journal of Food Protection* **52**: 144-147.

Park, CB, HS Kim and SC Kim (1998). "Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions." *Biochemical and biophysical research communications* **244**: 253-257.

Park, CB, KS Yi, K Matsuzaki, MS Kim and SC Kim (2000). "Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II." *Proceedings of the National Academy of Sciences of the United States of America* **97**(15): 8245-8250.

Patil, A, AL Hughes and G Zhang (2004). "Rapid evolution and diversification of mammalian alpha-defensins as revealed by comparative analysis of rodent and primate genes." *Physiological genomics* **20**(1): 1-11.

Péant, B, G LaPointe, C Gilbert, D Atlan, P Ward*, et al.* (2005). "Comparative analysis of the exopolysaccharide biosynthesis gene clusters from four strains of *Lactobacillus rhamnosus*." *Microbiology (Reading, England)* **151**: 1839-1851.

Peel, M, W Donachie and A Shaw (1988). "Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE and western blotting." *J Gen Microbiol* **134**: 2171-2178.

Percy, MG and A Gründling (2014). "Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria." *Annual review of microbiology*, 10.1146/annurev-micro-091213-112949: 81-100.

Perego, M, P Glaser, A Minutello, MA Strauch, K Leopold*, et al.* (1995). "Incorporation of D-Alanine into Lipoteichoic Acid and Wall Teichoic Acid in *Bacillus Subtilis* - Identification of Genes and Regulation." *J Biol Chem* **270**(26): 15598-15606.

Pérez-Dorado, I, S Galan-Bartual and Ja Hermoso (2012). "Pneumococcal surface proteins: when the whole is greater than the sum of its parts." *Molecular oral microbiology* **27**: 221-245.

Perlman, D and Bodanszk.M (1971). "Biosynthesis of Peptide Antibiotics." *Annu Rev Biochem* **40**: 449-&.

Personnic, N, S Bruck, M-A Nahori, A Toledo-Arana, G Nikitas*, et al.* (2010). "The stress-induced virulence protein InlH controls interleukin-6 production during murine listeriosis." *Infect Immun* **78**: 1979-1989.

Peschel, A (2002). "How do bacteria resist human antimicrobial peptides?" *Trends in Microbiology* **10**: 179-186.

Peschel, a, RW Jack, M Otto, LV Collins, P Staubitz*, et al.* (2001). "*Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine." *The Journal of experimental medicine* **193**: 1067-1076.

Peschel, a, M Otto, RW Jack, H Kalbacher, G Jung*, et al.* (1999). "Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides." *The Journal of Biological Chemistry* **274**: 8405-8410.

Peschel, A and H-G Sahl (2006). "The co-evolution of host cationic antimicrobial peptides and microbial resistance." *Nature reviews Microbiology* **4**: 529-536.

Peschel, A, C Vuong, M Otto and F Götz (2000). "The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes." *Antimicrob Agents Ch* **44**: 2845-2847.

Peters, BM, ME Shirtliff and MA Jabra-Rizk (2010). "Antimicrobial peptides: primeval molecules or future drugs?" *PLoS pathogens* **6**: e1001067.

Pierre, J, A Boisivon and L Gutmann (1990). "Alteration of PBP 3 entails resistance to imipenem in *Listeria monocytogenes*." *Antimicrob Agents Ch* **34**: 1695-1698.

Pilgrim, S, A Kolb-Mäurer, I Gentschev, W Goebel and M Kuhn (2003). "Deletion of the gene encoding p60 in *Listeria monocytogenes* leads to abnormal cell division and loss of actin-based motility." *Infect Immun* **71**: 3473-3484.

Pinto, D, C São-José, MA Santos and L Chambel (2013). "Characterization of two resuscitation promoting factors of *Listeria monocytogenes*." *Microbiology (Reading, England)* **159**: 1390-1401.

Pirie, JH (1927). "A new disease of veld rodents, "Tiger River disease" " *Publication of the South African Institute of Medical Research* **3**: 163-186.

Pirie, JH (1940). "The Genus Listerella Pirie." *Science* **91**(2364): 383.

Pistor, S, T Chakraborty, K Niebuhr, E Domann and J Wehland (1994). "The ActA protein of *Listeria monocytogenes* acts as a nucleator inducing reorganization of the actin cytoskeleton." *The EMBO journal* **13**: 758-763.

Pizarro-Cerdá, J, A Kühbacher and P Cossart (2012). "Entry of *Listeria monocytogenes* in mammalian epithelial cells: an updated view." *Cold Spring Harbor Perspectives in Medicine* **2**: 1-18.

Popowska, M (2004). "Analysis of the peptidoglycan hydrolases of *Listeria monocytogenes*: multiple enzymes with multiple functions." *Polish journal of microbiology / Polskie Towarzystwo Mikrobiologów = The Polish Society of Microbiologists* **53 Suppl**: 29-34.

Portnoy, DA, PS Jacks and DJ Hinrichs (1988). "Role of hemolysin for the intracellular growth of *Listeria monocytogenes*." *The Journal of experimental medicine* **167**: 1459-1471.

Poussin, MA and H Goldfine (2010). "Evidence for the involvement of ActA in maturation of the *Listeria monocytogenes* phagosome." *Cell Res* **20**(1): 109-112.

Promadej, N, F Fiedler, P Cossart, S Dramsi and S Kathariou (1999). "Cell wall teichoic acid glycosylation in *Listeria monocytogenes* serotype 4b requires *gtcA*, a novel, serogroup-specific gene." *J Bacteriol* **181**: 418-425.

Proud, D, SP Sanders and S Wiehler (2004). "Human rhinovirus infection induces airway epithelial cell production of human beta-defensin 2 both in vitro and in vivo." *J Immunol* **172**(7): 4637-4645.

Pucciarelli, MG, E Calvo, C Sabet, H Bierne, P Cossart*, et al.* (2005). "Identification of substrates of the *Listeria monocytogenes* sortases A and B by a non-gel proteomic analysis." *Proteomics* **5**: 4808-4817.

Qamar, A and D Golemi-Kotra (2012). "Dual Roles of FmtA in *Staphylococcus aureus* Cell Wall Biosynthesis and Autolysis." *Antimicrob Agents Ch* **56**(7): 3797-3805.

Radtke, AL, KL Anderson, MJ Davis, MJ DiMagno, JA Swanson*, et al.* (2011). "*Listeria monocytogenes* exploits cystic fibrosis transmembrane conductance regulator (CFTR) to escape the phagosome." *Proceedings of the National Academy of Sciences of the United States of America* **108**(4): 1633-1638.

Ramadurai, S, A Holt, LV Schafer, VV Krasnikov, DTS Rijkers*, et al.* (2010). "Influence of Hydrophobic Mismatch and Amino Acid Composition on the Lateral Diffusion of Transmembrane Peptides." *Biophys J* **99**(5): 1447-1454.

Ramamoorthy, A, S Thennarasu, AM Tan, K Gottipati, S Sreekumar*, et al.* (2006). "Deletion of all cysteines in tachyplesin I abolishes hemolytic activity and retains antimicrobial activity and lipopolysaccharide selective binding." *Biochemistry* **45**(20): 6529-6540.

Ramanathan, B, EG Davis, CR Ross and F Blecha (2002). "Cathelicidins: Microbicidal activity, mechanisms of action, and roles in innate immunity." *Microbes and Infection* **4**: 361-372.

Ramnath, M, M Beukes, K Tamura and JW Hastings (2000). "Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis." *Applied and environmental microbiology* **66**(7): 3098-3101.

Rechsteiner, M and SW Rogers (1996). "PEST sequences and regulation by proteolysis." *Trends Biochem Sci* **21**(7): 267-271.

Reichmann, NT, CP Cassona and A Gründling (2013). "Revised mechanism of D-alanine incorporation into cell wall polymers in Gram-positive bacteria." *Microbiology (Reading, England)* **159**: 1868-1877.

Reichmann, NT and A Gründling (2011). "Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes." *Fems Microbiol Lett* **319**: 97-105.

Reichmann, NT, C Piçarra Cassona, JM Monteiro, AL Bottomley, RM Corrigan*, et al.* (2014). "Differential localization of LTA synthesis proteins and their interaction with the cell division machinery in *Staphylococcus aureus*." *Mol Microbiol* **44**: 1-44.

Reis, O, S Sousa, A Camejo, V Villiers, E Gouin*, et al.* (2010). "LapB, a novel *Listeria monocytogenes* LPXTG surface adhesin, required for entry into eukaryotic cells and virulence." *The Journal of infectious diseases* **202**: 551-562.

Reiss, HJ, J Potel and A Krebs (1951). "[Granulomatosis infantiseptica; a general infection of infants and the newborn characterized by the presence of miliary granuloma]." *Z Gesamte Inn Med* **6**(15-16): 451-457.

Resnick, NM, WL Maloy, HR Guy and M Zasloff (1991). "A novel endopeptidase from *Xenopus* that recognizes alpha-helical secondary structure." *Cell* **66**(3): 541-554.

Ribet, D, M Hamon, E Gouin, MA Nahori, F Impens*, et al.* (2010). "*Listeria monocytogenes* impairs SUMOylation for efficient infection." *Nature* **464**(7292): 1192-1195.

Rigden, DJ, MJ Jedrzejas and MY Galperin (2003). "Amidase domains from bacterial and phage autolysins define a family of gamma-D,L-glutamate-specific amidohydrolases." *Trends Biochem Sci* **28**: 230-234.

Ritonja, A, M Kopitar, R Jerala and V Turk (1989). "Primary structure of a new cysteine proteinase inhibitor from pig leucocytes." *Febs Lett* **255**(2): 211-214.

Robey, M, W O'Connell and NP Cianciotto (2001). "Identification of *Legionella pneumophila rcp*, a *pagP*-like gene that confers resistance to cationic antimicrobial peptides and promotes intracellular infection." *Infect Immun* **69**(7): 4276-4286.

Rocourt, J, P Boerlin, F Grimont, C Jacquet and JC Piffaretti (1992). "Assignment of *Listeria-Grayi* and *Listeria-Murrayi* to a Single Species, *Listeria-Grayi*, with a Revised Description of *Listeria-Grayi*." *Int J Syst Bacteriol* **42**(1): 171-174.

Rocourt, J and C Buchrieser (2007). The Genus *Listeria* and *Listeria monocytogenes*. *In*., doi:10.1201/9781420015188.ch1**:** 1-20.

Rocourt, J and PAD Grimont (1983). "*Listeria-Welshimeri* Sp-Nov and *Listeria-Seeligeri* Sp-Nov." *Int J Syst Bacteriol* **33**(4): 866-869.

Rocourt, J, A Schrettenbrunner and HP Seeliger (1983). "Biochemical differentiation of the "*Listeria monocytogenes*" (sensu lato) genomic groups." *Annales de microbiologie* **134A**(1): 65-71.

Rogers, LA and EO Whittier (1928). "Limiting Factors in the Lactic Fermentation." *J Bacteriol* **16**(4): 211-229.

Rogne, P, G Fimland, J Nissen-Meyer and PE Kristiansen (2008). "Three-dimensional structure of the two peptides that constitute the two-peptide bacteriocin lactococcin G." *Biochim Biophys Acta* **1784**(3): 543-554.

Rohrl, J, D Yang, JJ Oppenheim and T Hehlgans (2010). "Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2." *J Immunol* **184**(12): 6688-6694.

Rosenberger, CM, RL Gallo and BB Finlay (2004). "Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular *Salmonella* replication." *Proceedings of the National Academy of Sciences of the United States of America* **101**: 2422-2427.

Ruhland, GJ and F Fiedler (1987). "Occurrence and biochemistry of lipoteichoic acids in the genus *Listeria*." *Syst Appl Microbiol* **9**: 40-46.

Ryan, MP, MC Rea, C Hill and RP Ross (1996). "An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147." *Applied and environmental microbiology* **62**(2): 612-619.

Saar-Dover, R, A Bitler, R Nezer, L Shmuel-Galia, A Firon*, et al.* (2012). "D-alanylation of lipoteichoic acids confers resistance to cationic peptides in group B streptococcus by increasing the cell wall density." *PLoS pathogens* **8**: e1002891.

Sabet, C, M Lecuit, D Cabanes, P Cossart and H Bierne (2005). "LPXTG protein InlJ, a newly identified internalin involved in *Listeria monocytogenes* virulence." *Infect Immun* **73**: 6912-6922.

Sabet, C, A Toledo-Arana, N Personnic, M Lecuit, S Dubrac*, et al.* (2008). "The *Listeria monocytogenes* virulence factor InlJ is specifically expressed *in vivo* and behaves as an adhesin." *Infect Immun* **76**: 1368-1378.

Samarin, S, S Romero, C Kocks, D Didry, D Pantaloni*, et al.* (2003). "How VASP enhances actin-based motility." *Journal of Cell Biology* **163**: 131-142.

Samuel, G and P Reeves (2003). "Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly." *Carbohydrate research* **338**: 2503-2519.

Sansom, MS, ID Kerr and IR Mellor (1991). "Ion channels formed by amphipathic helical peptides. A molecular modelling study." *European biophysics journal : EBJ* **20**(4): 229-240.

Sauvage, E, F Kerff, M Terrak, Ja Ayala and P Charlier (2008). "The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis." *Fems Microbiol Rev* **32**: 234-258.

Schaumburg, J, O Diekmann, P Hagendorff, S Bergmann, M Rohde*, et al.* (2004). "The cell wall subproteome of *Listeria monocytogenes*." *Proteomics* **4**: 2991-3006.

Schindler, CA and VT Schuhardt (1964). "Lysostaphin: A New Bacteriolytic Agent for the *Staphylococcus*." *Proceedings of the National Academy of Sciences of the United States of America* **51**: 414-421.

Schirner, K, J Marles-Wright, RJ Lewis and J Errington (2009). "Distinct and essential morphogenic functions for wall- and lipo-teichoic acids in *Bacillus subtilis*." *The EMBO journal* **28**: 830-842.

Schlag, M, R Biswas, B Krismer, T Kohler, S Zoll*, et al.* (2010). "Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl." *Mol Microbiol*, 10.1111/j.1365-2958.2009.07007.x: 1-10.

Schlech, WF, PM Lavigne, RA Bortolussi, AC Allen, EV Haldane*, et al.* (1983). "Epidemic listeriosis--evidence for transmission by food." *The New England journal of medicine* **308**: 203-206.

Schleifer, KH and O Kandler (1972). "Peptidoglycan types of bacterial cell walls and their taxonomic implications." *Bacteriological reviews* **36**: 407-477.

Schmid, MW, EYW Ng, R Lampidis, M Emmerth, M Walcher*, et al.* (2005). "Evolutionary history of the genus *Listeria* and its virulence genes." *Syst Appl Microbiol* **28**: 1-18.

Schmidtchen, A, IM Frick, E Andersson, H Tapper and L Bjorck (2002). "Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37." *Mol Microbiol* **46**(1): 157-168.

Schneewind, O, D Mihaylova-Petkov and P Model (1993). "Cell wall sorting signals in surface proteins of gram-positive bacteria." *The EMBO journal* **12**: 4803-4811.

Schneewind, O, P Model and Va Fischetti (1992). "Sorting of protein A to the staphylococcal cell wall." *Cell* **70**: 267-281.

Schnupf, P, J Hofmann, J Norseen, IJ Glomski, H Schwartzstein*, et al.* (2006). "Regulated translation of listeriolysin O controls virulence of *Listeria monocytogenes*." *Mol Microbiol* **61**(4): 999-1012.

Scocchi, M, B Skerlavaj, D Romeo and R Gennaro (1992). "Proteolytic cleavage by neutrophil elastase converts inactive storage proforms to antibacterial bactenecins." *Eur J Biochem* **209**(2): 589-595.

Scott, JR and TC Barnett (2006). "Surface proteins of gram-positive bacteria and how they get there." *Annual review of microbiology* **60**: 397-423.

Seeliger, HP (1981). "[Nonpathogenic listeriae: *L. innocua* sp. n. (Seeliger et Schoofs, 1977) (author's transl)]." *Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene 1 Abt Originale A, Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie = International journal of microbiology and hygiene A, Medical micro* **249**(4): 487-493.

Seeliger, HPR and K Höhne (1979). "Serotyping of *Listeria monocytogenes* and Related Species." *Methods in Microbiology* **13**: 31-49.

Seeliger, HPR and B Langer (1989). "Serological Analysis of the Genus *Listeria* - Its Values and Limitations." *Int J Food Microbiol* **8**(3): 245-248.

Seeliger, HPR, J Rocourt, A Schrettenbrunner, PAD Grimont and D Jones (1984). "*Listeria-Ivanovii* Sp-Nov." *Int J Syst Bacteriol* **34**(3): 336-337.

Selsted, ME, D Szklarek and RI Lehrer (1984). "Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes." *Infect Immun* **45**(1): 150-154.

Sengupta, D, H Leontiadou, AE Mark and SJ Marrink (2008). "Toroidal pores formed by antimicrobial peptides show significant disorder." *Biochim Biophys Acta* **1778**(10): 2308-2317.

Shahamat, M, A Seaman and M Woodbine (1980). "Survival of *Listeria monocytogenes* in high salt concentrations." *Zentralblatt fur Bakteriologie 1*

Abt Originale A: Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie **246**(4): 506-511.

Shai, Y and Z Oren (2001). "From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides." *Peptides* **22**(10): 1629-1641.

Shapiro, HM (2000). "Membrane potential estimation by flow cytometry." *Methods (San Diego, Calif)* **21**: 271-279.

Shatursky, O, AP Heuck, LA Shepard, J Rossjohn, MW Parker*, et al.* (1999). "The mechanism of membrane insertion for a cholesterol-dependent cytolysin: A novel paradigm for pore-forming toxins." *Cell* **99**(3): 293-299.

Shen, Y, M Naujokas, M Park and K Ireton (2000). "InIB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase." *Cell* **103**(30): 501-510.

Shi, J, CR Ross, TL Leto and F Blecha (1996). "PR-39, a proline-rich antibacterial peptide that inhibits phagocyte NADPH oxidase activity by binding to Src homology 3 domains of p47 phox." *Proceedings of the National Academy of Sciences of the United States of America* **93**(12): 6014-6018.

Sibelius, U, T Chakraborty, B Krogel, J Wolf, F Rose*, et al.* (1996). "The listerial exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C synergize to elicit endothelial cell phosphoinositide metabolism." *J Immunol* **157**(9): 4055-4060.

Sieprawska-Lupa, M, P Mydel, K Krawczyk, K Wojcik, M Puklo*, et al.* (2004). "Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases." *Antimicrob Agents Chemother* **48**(12): 4673-4679.

Silhavy, TJ, D Kahne and S Walker (2010). "The Bacterial Cell Envelope." **2**: 16.

Simon, R, U Priefer and A Pühler (1983). "A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria." *Bio/Technology* **1**: 784-791.

Singh, R, A Jamieson and P Cresswell (2008). "GILT is a critical host factor for *Listeria monocytogenes* infection." *Nature* **455**: 1244-1247.

Skarnes, RC and DW Watson (1957). "Antimicrobial factors of normal tissues and fluids." *Bacteriological reviews* **21**: 273-294.

Sleator, RD, J Wouters, CG Gahan, T Abee and C Hill (2001). "Analysis of the role of OpuC, an osmolyte transport system, in salt tolerance and virulence potential of *Listeria monocytogenes*." *Applied and environmental microbiology* **67**: 2692-2698.

Smith, GA, H Marquis, S Jones, NC Johnston, DA Portnoy*, et al.* (1995). "The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread." *Infect Immun* **63**: 4231-4237.

Soldo, B, V Lazarevic and D Karamata (2002). "*tagO* is involved in the synthesis of all anionic cell-wall polymers in *Bacillus subtilis* 168." *Microbiol-Sgm* **148**: 2079-2087.

Sorensen, O, K Arnljots, JB Cowland, DF Bainton and N Borregaard (1997). "The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils." *Blood* **90**(7): 2796-2803.

Sorensen, O, T Bratt, AH Johnsen, MT Madsen and N Borregaard (1999). "The human antibacterial cathelicidin, hCAP-18, is bound to lipoproteins in plasma." *J Biol Chem* **274**(32): 22445-22451.

Sorensen, OE, JB Cowland, K Theilgaard-Monch, L Liu, T Ganz*, et al.* (2003). "Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors." *J Immunol* **170**(11): 5583-5589.

Sorensen, OE, P Follin, AH Johnsen, J Calafat, GS Tjabringa*, et al.* (2001). "Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3." *Blood* **97**(12): 3951-3959.

Stavru, F, C Archambaud and P Cossart (2011). "Cell biology and immunology of *Listeria monocytogenes* infections: novel insights." *Immunological reviews* **240**: 160-184.

Steen, A, G Buist, KJ Leenhouts, M El Khattabi, F Grijpstra*, et al.* (2003). "Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents." *The Journal of biological chemistry* **278**: 23874-23881.

Steen, A, E Palumbo, M Deghorain, PS Cocconcelli, J Delcour*, et al.* (2005). "Autolysis of *Lactococcus lactis* is increased upon D-alanine depletion of peptidoglycan and lipoteichoic acids." *J Bacteriol* **187**(1): 114-124.

Steinweg, C, CT Kuenne, A Billion, MA Mraheil, E Domann*, et al.* (2010). "Complete Genome Sequence of *Listeria seeligeri*, a Nonpathogenic Member of the Genus *Listeria*." *J Bacteriol* **192**(5): 1473-1474.

Stinavage, P, LE Martin and JK Spitznagel (1989). "O antigen and lipid A phosphoryl groups in resistance of *Salmonella typhimurium* LT-2 to nonoxidative killing in human polymorphonuclear neutrophils." *Infect Immun* **57**(12): 3894-3900.

Suarez, M, B Gonzalez-Zorn, Y Vega, I Chico-Calero and JA Vazquez-Boland (2001). "A role for ActA in epithelial cell invasion by *Listeria monocytogenes*." *Cellular microbiology* **3**(12): 853-864.

Subbalakshmi, C and N Sitaram (1998). "Mechanism of antimicrobial action of indolicidin." *Fems Microbiol Lett* **160**(1): 91-96.

Sutcliffe, IC and DJ Harrington (2002). "Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes." *Microbiology (Reading, England)* **148**: 2065-2077.

Sutcliffe, IC and RR Russell (1995). "Lipoproteins of gram-positive bacteria." *J Bacteriol* **177**: 1123-1128.

Swaminathan, B and P Gerner-Smidt (2007a). "The epidemiology of human listeriosis." *Microbes and Infection* **9**: 1236-1243.

Swaminathan, B and P Gerner-Smidt (2007b). The epidemiology of human listeriosis. Microbes and Infection. **9:** 1236-1243.

Swoboda, JG, J Campbell, TC Meredith and S Walker (2010). "Wall teichoic acid function, biosynthesis, and inhibition." *Chembiochem : a European journal of chemical biology* **11**: 35-45.

Tam, R and MH Saier (1993). "Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria." *Microbiological reviews* **57**: 320-346.

Tang, P, I Rosenshine, P Cossart and BB Finlay (1996). "Listeriolysin O activates mitogen-activated protein kinase in eukaryotic cells." *Infect Immun* **64**(6): 2359-2361.

Tang, YQ, J Yuan, G Osapay, K Osapay, D Tran*, et al.* (1999). "A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins." *Science* **286**(5439): 498-502.

Tareq, FS, MA Lee, HS Lee, YJ Lee, JS Lee*, et al.* (2014). "Gageotetrins A-C, Noncytotoxic Antimicrobial Linear Lipopeptides from a Marine Bacterium Bacillus subtilis." *Org Lett* **16**(3): 928-931.

Thedieck, K, T Hain, W Mohamed, BJ Tindall, M Nimtz*, et al.* (2006). "The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*." *Mol Microbiol* **62**: 1325-1339.

Theriot, JA, J Rosenblatt, DA Portnoy, PJ Goldschmidt-Clermont and TJ Mitchisont (1994). "Involvement of profilin in the actin-based motility of *L. monocytogenes* in cells and in cell-free extracts." *Cell* **76**: 505-517.

Tipper, DJ and JL Strominger (1965). "Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine." *Proceedings of the National Academy of Sciences of the United States of America* **54**: 1133-1141.

Toledo-Arana, A, O Dussurget, G Nikitas, N Sesto, H Guet-Revillet*, et al.* (2009). "The *Listeria* transcriptional landscape from saprophytism to virulence." *Nature* **459**: 950-956.

Ton-That, H, KF Faull and O Schneewind (1997). "Anchor structure of staphylococcal surface proteins. A branched peptide that links the carboxyl terminus of proteins to the cell wall." *The Journal of biological chemistry* **272**: 22285-22292.

Ton-That, H, G Liu, SK Mazmanian, KF Faull and O Schneewind (1999). "Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif." *Proceedings of the National Academy of Sciences of the United States of America* **96**: 12424-12429.

Tonetti, M, L Sturla, A Bisso, D Zanardi, U Benatti*, et al.* (1998). "The metabolism of 6-deoxyhexoses in bacterial and animal cells." *Biochimie* **80**(11): 923-931.

Torii, M, EA Kabat and AE Bezer (1964). "Separation of teichoic acid of *Staphylococcus aureus* into two immunologically distinct specific polysaccharides with alpha- and beta-N-acetylglucosaminyl linkages respectively. Antigenicity of teichoic acids in man." *The Journal of experimental medicine* **120**: 13-29.

Tran, AX, JD Whittimore, PB Wyrick, SC McGrath, RJ Cotter*, et al.* (2006). "The lipid A 1-phosphatase of *Helicobacter pylori* is required for resistance to the antimicrobial peptide polymyxin." *J Bacteriol* **188**(12): 4531-4541.

Travier, L, S Guadagnini, E Gouin, A Dufour, V Chenal-Francisque*, et al.* (2013). "ActA promotes *Listeria monocytogenes* aggregation, intestinal colonization and carriage." *PLoS pathogens* **9**: e1003131.

Trost, M, D Wehmhöner, U Kärst, G Dieterich, J Wehland*, et al.* (2005). "Comparative proteome analysis of secretory proteins from pathogenic and nonpathogenic *Listeria* species." *Proteomics* **5**: 1544-1557.

Tsukioka, Y, Y Yamashita, T Oho, Y Nakano and T Koga (1997). "Biological function of the dTDP-rhamnose synthesis pathway in *Streptococcus mutans*." *J Bacteriol* **179**: 1126-1134.

Tweten, RK, MW Parker and AE Johnson (2001). "The cholesterol-dependent cytolysins." *Curr Top Microbiol* **257**: 15-33.

Uchikawa, K, I Sekikawa and I Azuma (1986a). "Structural studies on teichoic acids in cell walls of several serotypes of Listeria monocytogenes." *Journal of biochemistry* **99**: 315-327.

Uchikawa, K-i, I Sekikawa and I Azuma (1986b). "Structural studies on lipoteichoic acids from four *Listeria* strains." *J Bacteriol* **168**: 115-122.

Uematsu, N and K Matsuzaki (2000). "Polar angle as a determinant of amphipathic alpha-helix-lipid interactions: a model peptide study." *Biophys J* **79**(4): 2075-2083.

Ullmann, WW and Ja Cameron (1969). "Immunochemistry of the cell walls of *Listeria monocytogenes*." *J Bacteriol* **98**: 486-493.

Umeda, A, S Yokoyama, T Arizono and K Amako (1992). "Location of peptidoglycan and teichoic acid on the cell wall surface of *Staphylococcus aureus* as determined by immunoelectron microscopy." *Journal of electron microscopy* **41**: 46-52.

Vadyvaloo, V, S Arous, A Gravesen, Y Héchard, R Chauhan-Haubrock*, et al.* (2004). "Cell-surface alterations in class IIa bacteriocin-resistant *Listeria monocytogenes* strains." *Microbiology (Reading, England)* **150**: 3025-3033.

Valore, EV and T Ganz (1992). "Posttranslational processing of defensins in immature human myeloid cells." *Blood* **79**(6): 1538-1544.

Valore, EV, E Martin, SS Harwig and T Ganz (1996). "Intramolecular inhibition of human defensin HNP-1 by its propiece." *The Journal of clinical investigation* **97**(7): 1624-1629.

van Belkum, MJ, J Kok, G Venema, H Holo, IF Nes*, et al.* (1991). "The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner." *J Bacteriol* **173**(24): 7934-7941.

van de Wetering, JK, M van Eijk, LMG van Golde, T Hartung, JAG van Strijp*, et al.* (2001). "Characteristics of surfactant protein A and D binding to lipoteichoic acid and peptidoglycan, 2 major cell wall components of Gram-positive bacteria." *J Infect Dis* **184**(9): 1143-1151.

van der Wal, FJ, J Luirink and B Oudega (1995). "Bacteriocin release proteins: mode of action, structure, and biotechnological application." *Fems Microbiol Rev* **17**(4): 381-399.

van Heijenoort, J (1998). "Assembly of the monomer unit of bacterial peptidoglycan." *Cellular and molecular life sciences : CMLS* **54**(4): 300-304.

van Heusden, HE, B de Kruijff and E Breukink (2002). "Lipid II induces a transmembrane orientation of the pore-forming peptide lantibiotic nisin." *Biochemistry* **41**(40): 12171-12178.

Van Tyne, D, MJ Martin and MS Gilmore (2013). "Structure, Function, and Biology of the *Enterococcus faecalis* Cytolysin." *Toxins* **5**(5): 895-911.

Vandamme, D, B Landuyt, W Luyten and L Schoofs (2012). "A comprehensive summary of LL-37, the factoctum human cathelicidin peptide." *Cellular immunology* **280**: 22-35.

Vazquez-Boland, JA, C Kocks, S Dramsi, H Ohayon, C Geoffroy*, et al.* (1992). "Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread." *Infect Immun* **60**: 219-230.

Vazquez-Boland, JA, M Kuhn, P Berche, T Chakraborty, G Dominguez-Bernal*, et al.* (2001). "*Listeria* pathogenesis and molecular virulence determinants." *Clinical microbiology reviews* **14**(3): 584-640.

Veiga, P, C Bulbarela-Sampieri, S Furlan, A Maisons, M-P Chapot-Chartier*, et al.* (2007). "SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis." *The Journal of biological chemistry* **282**: 19342-19354.

Vergara-Irigaray, M, T Maira-Litran, N Merino, GB Pier, JR Penades*, et al.* (2008). "Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the *Staphylococcus aureus* cell surface." *Microbiology* **154**(Pt 3): 865-877.

Vicente, MF, J Berenguer, MA de Pedro, JC Pérez-Diaz and F Baquero (1990). "Penicillin binding proteins in *Listeria monocytogenes*." *Acta Microbiol Hung* **37**: 227-231.

Vollmer, W (2008). "Structural variation in the glycan strands of bacterial peptidoglycan." *Fems Microbiol Rev* **32**: 287-306.

Vollmer, W, D Blanot and Ma De Pedro (2008a). "Peptidoglycan structure and architecture." *Fems Microbiol Rev* **32**: 149-167.

Vollmer, W, B Joris, P Charlier and S Foster (2008b). "Bacterial peptidoglycan (murein) hydrolases." *Fems Microbiol Rev* **32**: 259-286.

Walter, J, DM Loach, M Alqumber, C Rockel, C Hermann*, et al.* (2007). "D-alanyl ester depletion of teichoic acids in *Lactobacillus reuteri* 100-23 results in impaired colonization of the mouse gastrointestinal tract." *Environmental microbiology* **9**(7): 1750-1760.

Wang, G, LF Lo, LS Forsberg and RJ Maier (2012). "*Helicobacter pylori* peptidoglycan modifications confer lysozyme resistance and contribute to survival in the host." *mBio* **3**(6): e00409-00412.

Wang, GS, X Li and Z Wang (2009). "APD2: the updated antimicrobial peptide database and its application in peptide design." *Nucleic Acids Res* **37**: D933-D937.

Wang, L and M Lin (2007). "Identification of IspC, an 86-kilodalton protein target of humoral immune response to infection with *Listeria monocytogenes* serotype 4b, as a novel surface autolysin." *J Bacteriol* **189**: 2046-2054.

Wang, L and M Lin (2008). "A novel cell wall-anchored peptidoglycan hydrolase (autolysin), IspC, essential for *Listeria monocytogenes* virulence: genetic and proteomic analysis." *Microbiology (Reading, England)* **154**: 1900-1913.

Ward, JB (1973). "The chain length of the glycans in bacterial cell walls." *The Biochemical journal* **133**(2): 395-398.

Ward, JB (1981). "Teichoic and teichuronic acids: biosynthesis, assembly, and location." *Microbiological reviews* **45**: 211-243.

Webb, AJ, M Karatsa-Dodgson and A Gründling (2009). "Two-enzyme systems for glycolipid and polyglycerolphosphate lipoteichoic acid synthesis in *Listeria monocytogenes*." *Mol Microbiol* **74**: 299-314.

Weidenmaier, C, JF Kokai-Kun, SA Kristian, T Chanturiya, H Kalbacher*, et al.* (2004). "Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections." *Nature medicine* **10**: 243-245.

Weidenmaier, C and A Peschel (2008). "Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions." *Nature reviews Microbiology* **6**: 276-287.

Weidenmaier, C, A Peschel, Y-Q Xiong, Sa Kristian, K Dietz*, et al.* (2005). "Lack of wall teichoic acids in *Staphylococcus aureus* leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis." *The Journal of infectious diseases* **191**: 1771-1777.

Weiglein, I, W Goebel, J Troppmair, UR Rapp, A Demuth*, et al.* (1997). "*Listeria monocytogenes* infection of HeLa cells results in listeriolysin O-mediated transient activation of the Raf-MEK-MAP kinase pathway." *Fems Microbiol Lett* **148**(2): 189-195.

Weis, J and HP Seeliger (1975). "Incidence of *Listeria monocytogenes* in nature." *Applied microbiology* **30**: 29-32.

Welch, MD, J Rosenblatt, J Skoble, DA Portnoy and TJ Mitchison (1998). "Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin filament nucleation." *Science* **281**(5373): 105-108.

Wendlinger, G, MJ Loessner and S Scherer (1996). "Bacteriophage receptors on *Listeria monocytogenes* cells are the N-acetylglucosamine and rhamnose substituents of teichoic acids or the peptidoglycan itself." *Microbiology (Reading, England)* **142 (Pt 4**: 985-992.

Wiedemann, I, E Breukink, C van Kraaij, OP Kuipers, G Bierbaum*, et al.* (2001). "Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity." *J Biol Chem* **276**(3): 1772-1779.

Wiesner, J and A Vilcinskas (2010). "Antimicrobial peptides: the ancient arm of the human immune system." *Virulence* **1**: 440-464.

Wilson, CL, AJ Ouellette, DP Satchell, T Ayabe, YS Lopez-Boado*, et al.* (1999). "Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense." *Science* **286**(5437): 113-117.

Wobser, D, L Ali, E Grohmann, J Huebner and T Sakinc (2014). "A novel role for D-alanylation of lipoteichoic acid of *Enterococcus faecalis* in urinary tract infection." *PloS one* **9**(10): e107827.

Wong, JH, L Xia and TB Ng (2007). "A review of defensins of diverse origins." *Curr Protein Pept Sci* **8**(5): 446-459.

Wu, Z, DM Hoover, D Yang, C Boulegue, F Santamaria*, et al.* (2003). "Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3." *Proceedings of the National Academy of Sciences of the United States of America* **100**(15): 8880-8885.

Wuenscher, MD, S Köhler, A Bubert, U Gerike and W Goebel (1993). "The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity." *J Bacteriol* **175**: 3491-3501.

Xiao, Q, X Jiang, KJ Moore, Y Shao, H Pi*, et al.* (2011). "Sortase independent and dependent systems for acquisition of haem and haemoglobin in *Listeria monocytogenes*." *Mol Microbiol* **80**: 1581-1597.

Yamada, S, M Sugai, H Komatsuzawa, S Nakashima, T Oshida*, et al.* (1996). "An autolysin ring associated with cell separation of *Staphylococcus aureus*." *J Bacteriol* **178**(6): 1565-1571.

Yang, D, Q Chen, O Chertov and JJ Oppenheim (2000). "Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells." *Journal of leukocyte biology* **68**(1): 9-14.

Yang, D, O Chertov, SN Bykovskaia, Q Chen, MJ Buffo*, et al.* (1999). "Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6." *Science* **286**(5439): 525-528.

Yang, L, TA Harroun, TM Weiss, L Ding and HW Huang (2001). "Barrel-stave model or toroidal model? A case study on melittin pores." *Biophys J* **81**(3): 1475-1485.

Yang, SC, CH Lin, CT Sung and JY Fang (2014). "Antibacterial activities of bacteriocins: Application in foods and pharmaceuticals." *Frontiers in Microbiology* **5**: 1-10.

Yau, WM, WC Wimley, K Gawrisch and SH White (1998). "The preference of tryptophan for membrane interfaces." *Biochemistry* **37**(42): 14713-14718.

Yeaman, MR and NY Yount (2003). "Mechanisms of antimicrobial peptide action and resistance." *Pharmacological reviews* **55**: 27-55.

Yoshikawa, H, I Kawamura, M Fujita, H Tsukada, M Arakawa*, et al.* (1993). "Membrane Damage and Interleukin-1 Production in Murine Macrophages Exposed to Listeriolysin O." *Infect Immun* **61**(4): 1334-1339.

Yoshikawa, Y, M Ogawa, T Hain, T Chakraborty and C Sasakawa (2009). "*Listeria monocytogenes* ActA is a key player in evading autophagic recognition." *Autophagy* **5**: 1220-1221.

Yother, J and JM White (1994). "Novel surface attachment mechanism of the *Streptococcus pneumoniae* protein PspA." *J Bacteriol* **176**: 2976-2985.

Yount, NY, MS Wang, J Yuan, N Banaiee, AJ Ouellette*, et al.* (1995). "Rat neutrophil defensins. Precursor structures and expression during neutrophilic myelopoiesis." *J Immunol* **155**(9): 4476-4484.

Zalevsky, J, I Grigorova and RD Mullins (2001). "Activation of the Arp2/3 complex by the *Listeria* ActA protein. acta binds two actin monomers and three subunits of the Arp2/3 complex." *J Biol Chem* **276**: 3468-3475.

Zanetti, M (2005). "The role of cathelicidins in the innate host defenses of mammals." *Current Issues in Molecular Biology* **7**: 179-196.

Zanetti, M, G Del Sal, P Storici, C Schneider and D Romeo (1993). "The cDNA of the neutrophil antibiotic Bac5 predicts a pro-sequence homologous to a cysteine proteinase inhibitor that is common to other neutrophil antibiotics." *J Biol Chem* **268**(1): 522-526.

Zanetti, M, R Gennaro and D Romeo (1995). "Cathelicidins: A novel protein family with a common proregion and a variable C-terminal antimicrobial domain." *Febs Lett* **374**: 1-5.

Zanetti, M, L Litteri, R Gennaro, H Horstmann and D Romeo (1990). "Bactenecins, defense polypeptides of bovine neutrophils, are generated from precursor molecules stored in the large granules." *The Journal of cell biology* **111**(4): 1363-1371.

Zanetti, M, L Litteri, G Griffiths, R Gennaro and D Romeo (1991). "Stimulus-induced maturation of probactenecins, precursors of neutrophil antimicrobial polypeptides." *J Immunol* **146**(12): 4295-4300.

Zasloff, M (2002). "Antimicrobial peptides of multicellular organisms." *Nature* **415**(6870): 389-395.

Zawadzka-Skomial, J, Z Markiewicz, M Nguyen-Distèche, B Devreese, J-M Frère*, et al.* (2006). "Characterization of the bifunctional glycosyltransferase/acyltransferase penicillin-binding protein 4 of *Listeria monocytogenes*." *J Bacteriol* **188**: 1875-1881.

Zayni, S, K Steiner, A Pföstl, A Hofinger, P Kosma*, et al.* (2007). "The dTDP-4-dehydro-6-deoxyglucose reductase encoding *fcd* gene is part of the surface layer glycoprotein glycosylation gene cluster of *Geobacillus tepidamans* GS5-97T." *Glycobiology* **17**: 433-443.

Zemansky, J, BC Kline, JJ Woodward, JH Leber, H Marquis*, et al.* (2009). "Development of a mariner-based transposon and identification of *Listeria*

monocytogenes determinants, including the peptidyl-prolyl isomerase PrsA2, that contribute to its hemolytic phenotype." *J Bacteriol* **191**: 3950-3964.

Zenewicz, LA and H Shen (2007). "Innate and adaptive immune responses to *Listeria monocytogenes*: a short overview." *Microbes and infection / Institut Pasteur* **9**(10): 1208-1215.

Zhao, C, T Ganz and RI Lehrer (1995). "Structures of genes for two cathelin-associated antimicrobial peptides: prophenin-2 and PR-39." *Febs Lett* **376**(3): 130-134.

Zhou, Z, AA Ribeiro, S Lin, RJ Cotter, SI Miller*, et al.* (2001). "Lipid A modifications in polymyxin-resistant *Salmonella typhimurium*: PMRA-dependent 4-amino-4-deoxy-L-arabinose, and phosphoethanolamine incorporation." *J Biol Chem* **276**(46): 43111-43121.

Zimmermann, GR, P Legault, ME Selsted and A Pardi (1995). "Solution structure of bovine neutrophil beta-defensin-12: the peptide fold of the beta-defensins is identical to that of the classical defensins." *Biochemistry* **34**(41): 13663-13671.

Zulianello, L, C Canard, T Kohler, D Caille, JS Lacroix*, et al.* (2006). "Rhamnolipids are virulence factors that promote early infiltration of primary human airway epithelia by *Pseudomonas aeruginosa*." *Infect Immun* **74**(6): 3134-3147.

CHAPTER VI

APPENDICES

This chapter includes a copy of the published version of the following publications:

- **Carvalho F**, Atilano ML, Pombinho R, Covas G, Gallo R, Filipe SR, Sousa S, Cabanes D (2015). L-rhamnosylation of *Listeria monocytogenes* wall teichoic acids promotes resistance to antimicrobial peptides by delaying interaction with the membrane. *PLoS Pathog* **11**(5):e1004919.

- **Carvalho F**, Sousa S, Cabanes D (2014). How *Listeria monocytogenes* organizes its surface for virulence. *Front Cell Infect Microbiol* **4**(48).

- Camejo, A, Buchrieser C, Couvé E, **Carvalho F**, Reis O, Ferreira P, Sousa S, Cossart P, Cabanes D (2009). *In vivo* transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. *PLoS Pathog* **5**:e1000449.