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Molecular characterization of newly identified *Listeria monocytogenes* virulence regulators

Jorge Nuno Pinheiro

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Jorge Nuno Martins Campos Pinheiro

D.ICBAS **2017**

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

JORGE NUNO MARTINS CAMPOS PINHEIRO

MOLECULAR CHARACTERIZATION OF NEWLY IDENTIFIED *LISTERIA MONOCYTOGENES* VIRULENCE REGULATORS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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According to the relevant national legislation, the author declares that this thesis includes data from the publication indicated below. The author participated actively in the conception and execution of the work that originated that data, as well as in their interpretation, discussion and writing.

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À mãe, ao pai

À avó, à famelga

À Sofia

À “Irmandade do Chamade”

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Abstract

Listeria monocytogenes is a bacterial foodborne pathogen that causes listeriosis, an invasive and potentially fatal infection in susceptible animals and humans. To infect, survive and proliferate in the host *L. monocytogenes* requires tight and coordinate networks of virulence regulation. In this work, we identify and characterize MouR and Lmo0443, two novel regulators of *L. monocytogenes,* and through RNAseq transcriptomic analysis we identify the genes under their transcriptional control. Importantly, we describe MouR as the central activator of the Agr system (*agrBDCA*). We also reveal how MouR binds to the promoter DNA of the *agr* locus, modulates biofilm formation and chitinase activity and is required for full virulence in the mouse infection model. By resolving its crystal structure, we were able to classify MouR as a dimeric DNA-binding VanR (FadR subfamily) regulator of the GntR family of transcriptional factors. Lmo0443 is shown here as the transcriptional repressor of *lmo0444*, an uncharacterized gene from the stress survival islet 1 (SSI-1) which is implicated in stress tolerance. Here we demonstrate that Lmo0443 is required for successful mouse infection, revealing its role in virulence, and propose a novel role for Lmo0443 and Lmo0444 in cell wall dynamics. In this work we also demonstrate how *L. monocytogenes* regulates virulence through control of protein secretion. The ESX-1 secretion system, responsible for the secretion of WXG100 proteins and implicated in virulence of *Mycobacterium tuberculosis,* was previously thought to be dispensable for *L. monocytogenes* virulence. Here we reveal how the secretion of EsxA by the ESX-1 system has a detrimental effect on *L. monocytogenes* virulence.

Resumo

Listeria monocytogenes é um agente bacteriano alimentar patogénico causador da listeriose, uma infeção invasiva e potencialmente fatal em animais e humanos suscetíveis. A infeção, sobrevivência e proliferação da *L. monocytogenes* no hospedeiro requer uma rede bem coordenada de regulação de virulência. Neste trabalho identificamos e caracterizamos MouR e Lmo0443, dois novos reguladores da *L. monocytogenes*, e através de análise transcriptómica por RNAseq identificamos os genes sob o controlo transcripcional de cada regulador. De importante realce, descrevemos MouR como o ativador central do sistema Agr (*agrBDCA*). Revelamos também como MouR se liga ao DNA promotor do locus *agr,* como modula a formação de biofilme e atividade da quitinase e como é necessário para a completa virulência no modelo de infeção do murganho. Caracterizando a sua estrutura em cristal, fomos capazes de classificar MouR como um regulador pertencente à classe VanR (subfamília FadR) da família de fatores de transcrição GntR e capaz de ligar DNA. Mostrámos como Lmo0443 é um repressor da transcrição do gene *lmo0444*, um componente não caracterizado do agregado de genes designado “stress survival islet 1 (SSI-1)”, o qual está relacionado com tolerância a stress. Nós demonstrámos o papel do Lmo0443 na virulência revelando, de igual modo, como ele é necessário para a infeção bem-sucedida em murganho e propusemos ainda um novo papel para o Lmo0443 e Lmo0444, relacionado com a parede celular. Neste trabalho demonstramos também, como a *L. monocytogenes* regula a virulência ao nível da secreção de proteínas. O sistema de secreção ESX-1, responsável pela secreção de proteínas do tipo WXG100 e relacionado com a virulência em *Mycobacterium tuberculosis,* era tido como dispensável para a virulência em *L. monocytogenes*. Revelámos aqui como a secreção da proteína EsxA, através do sistema ESX-1, tem um efeito prejudicial na virulência em *L. monocytogenes*.

List of abbreviations

ABC – ATP-binding cassette

Agr – accessory Gene Regulator

AIP – auto-inducing peptide

Ala – alanine

Arg – arginine

Arp2/3 – actin-related proteins 2 and 3

ASA – accessible surface area

Asn – asparagine

ATP – adenosine triphosphate

ATPase – adenosine triphosphatase

BCAA – branch-chained amino acids

BHI – brain and heart infusion

BSA – bovine serum albumin

CDC – cholesterol-dependent cytolysin

cDNA – complementary DNA

CFU – colony-forming unit

Crp – cAMP receptor protein

C-terminal – carboxy-terminal

Cys – cysteine

DNA – deoxyribonucleic acid

DNase – deoxyribonuclease

dsDNA – double-stranded DNA

DTT – dithiothreitol

ECF – extracytoplasmic function

EDTA – ethylenediamine tetracetic acid

EMSA – electrophoretic mobility shift assay

E-O – effector binding/oligomerization

eSTK – eukaryotic-like serine-threonine kinase

eSTP – eukaryotic-like serine-threonine phosphatase

FBS – fetal bovine serum

FCD – FadR C-terminal domain

FEA – flagellar export apparatus

Fnr – fumarate and nitrate reduction

FPE – fimbrilin protein exporter

FPKM – fragments per kilobase of transcript per million mapped reads

G+C – guanine-cytosine content

gC1qR – receptor for the globular component of complement C1q

Gln – glutamine

Glu – glutamate

Gly – glycine

GM-CSF – granulocyte-macrophage colony-stimulating factor

GW – glycine-tryptophan dipeptide

HEPA – high efficiency particulate air

His – histidine

Hph – hydrophobic

HPK – histidine protein kinase

HTH – helix-turn-helix

Hyd – hydrogen bond

IL – Interleukin

Ile – isoleucine

iNOS – inducible nitric oxide synthase

IR – inter-repeat

LB – lysogeny broth

LCP – LytR-CpsA-Psr protein

LIPI-1 – *Listeria* pathogenicity island 1

LisCVs – *Listeria*-Containing Vacuoles

LLO – listeriolysin O

Lm – *Listeria monocytogenes*

LRR – leucine-rich repeat

LTA – lipoteichoic acid

Lys – lysine

mRNA – messenger RNA

NF-κB – nuclear factor kappa B

NO – nitric oxide

N-terminal – amino-terminal

OD – optical density

ORF – open reading frame

PBS – phosphate-buffered saline

PC-PLC – phosphatidylcholine-specific phospholipase C

PCR – polymerase chain reaction

PE – proline-glutamic acid

Phe – phenylalanine

PI-PLC – phosphatidylinositol-specific phospholipase C

PPE – proline-proline-glutamic acid

PrfA – positive regulatory factor A

RMSD – root-mean-square deviation

RNA – ribonucleic acid

ROS – reactive oxygen species

RNAseq – RNA sequencing

rRNA – ribosomal RNA

RT-qPCR – reverse transcription quantitative real-time PCR

SAD – single-wavelength anomalous dispersion

Sal – salt bridge

SAM – S-adenosylmethionine

SD – Shine-Dalgarno

SDS – sodium dodecylsulfate

SeMet – selenomethionine

Ser – serine

sRNA – short RNA

SSI – stress survival islet

SUMO – small ubiquitin-like modifier protein

Tat – twin arginine translocation

TF – transcription factors

Thr – threonine

Trp – tryptophan

UTR – untranslated region

Val – valine

WT – wild type

WTA – wall teichoic acid

CHAPTER I – INTRODUCTION

Listeria monocytogenes

Historical overview and taxonomy

In 1924 E. G. D. Murray, R. A. Webb and M. B. R. Swann documented the isolation of an undescribed bacterial species responsible for a septicemic disease that was affecting rabbits and guinea pigs in England (Murray et al., 1926). Claiming that its most striking characteristic was a large mononuclear leukocytosis, the authors named this species *Bacterium monocytogenes*. It was not until several years later that this bacterium was reported to be linked to cases of meningitis in humans (Burn, 1935). In 1940 *Listeria monocytogenes* (*Lm*) was suggested as the denomination for this genus and species, in honor of the pioneer in surgical sterility Joseph Lister (Pirie, 1940). For the following years infection by *Lm* was considered rare and sporadic and the route of infection was not determined. It was more than forty years later that a report linked a serious outbreak to the ingestion of coleslaw contaminated with *Lm*, finally establishing it as a foodborne pathogen (Schlech et al., 1983). Interestingly, it is possible that an isolate from a meningitis patient back in 1921 might be oldest *Lm* strain ever collected, actually preceding the species identification (Dumont and Cotoni, 1921).

Lm is one of several species of the genus *Listeria*, from the Listeriaceae family, Bacillalles order, Bacilli class, Firmicutes phylum of the Bacteria kingdom. Other notable members of the Bacillalles order include the genera *Bacillus and Staphylococcus.* Whereas today the *Listeria* genus is composed of seventeen different species, it was not until more than 25 years after the species name *Lm* was defined in 1940 that another *Listeria* species, *Listeria grayi* (Larsen and Seeliger, 1966), was discovered which was shortly followed by the discovery of *Listeria innocua* (Seeliger and Listerien, 1981), *Listeria welshimeri*, *Listeria seeligeri* (Rocourt and Grimont, 1983) and *Listeria ivanovii* (Seeliger et al., 1984). For more than another twenty five years the genus remained unchanged until recently a new wave of species has been discovered: *Listeria marthii* (Graves et al., 2010), *Listeria rocourtiae* (Leclercq et al., 2010), *Listeria fleischmannii* (Bertsch et al., 2013), *Listeria weihenstephanensis* (Halter et al., 2013), *Listeria floridensis*, *Listeria aquatica*, *Listeria cornellensis*, *Listeria riparia* and *Listeria grandensis* (den Bakker et al., 2014), *Listeria booriae* and *Listeria newyorkensis* (Weller et al., 2015). *Lm* and *L. ivanovii* are the only two pathogenic species, both capable of causing disease in animal hosts while *Lm* is the only human pathogen (Cossart, 2007).

The identification of *Listeria* species was initially based on traditional microbiologic and chemical assays with the inclusion of more modern molecular approaches later on. These typically included: batteries of biochemical tests to determine carbohydrate utilization; hemolysis activity; motility testing; growth on selective agar medium; DNA probing for colony

formation; antibiotic susceptibility tests; 16S DNA sequencing; DNA–DNA hybridization; multilocus enzyme electrophoresis and serotyping (Gorski, 2008; Liu et al., 2008).

Serotyping of *Lm* strains became an important diagnosis tool for epidemiological purposes. The serotype of a *Listeria* strain is defined on the basis of its somatic (O) and flaggelar (H) antigens that have group specificity and allows their identification by immunological assays. At least thirteen different serotypes can be identified for *Lm* species: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. With the support of genetic subtyping the serotypes of *Lm* were separated into four different lineages. Lineage I includes the 1/2b, 3b, 3c and 4b serotypes, lineage II includes the 1/2a, 1/2c, 3a, lineage III includes the serotypes 4a, 4b and 4c and lineage IV includes rare strains of the 4a, 4b and 4c serotypes (Gorski, 2008; Orsi et al., 2011; Seeliger et al., 1979).

The identification and characterization of *Lm* species saw a great leap forward in the past decades with the scientific and technological advances in the genomics field, which greatly contributed to better comprehension of *Lm* phylogenetic evolution. The development of new and more advanced DNA sequencing techniques led to the first whole-genome sequencing of two *Listeria* species *Lm* EGD-e and *L. innocua* CLIP 11262 (Glaser et al., 2001). This was shortly followed by a boom in genome sequencing of *Listeria* species and the NCBI Genome database currently holds more than two thousand entries of sequences for *Lm* strains alone.

Today, *Lm* is the one of the best well-studied foodborne pathogens and has truly become an outstanding multidisciplinary model for advancement in the fields of microbiology and infection (e.g. identification and function characterization of virulence factors) and cell biology (e.g. understanding of cellular pathways and discovery of new molecular mechanism) (Cossart, 2011; Cossart and Lebreton, 2014). Its application in medicine has come a long way from induction of antibody-independent cell-mediated immune response (Lane and Unanue, 1972) to the very recent cancer vaccine treatment (Bolhassani et al., 2017; Jahangir et al., 2017; Rothman and Paterson, 2013).

General features and physiology

Lm is a Gram-positive, facultative anaerobic and facultative intracellular, catalase positive and oxidase negative pathogenic bacterium that is unable to form spores or capsule. Cells are small rods (up to 2 µm long and 0.5 µm in diameter) with round ends, usually found as single cells or short chains. Cells have flagella-dependent motility at 20°C-30°C but at temperatures higher than 37°C (physiological temperature) flagella expression is inhibited (Gründling et al., 2004; Peel et al., 1988; Rocourt and Buchrieser, 2007). *Lm* cells display both

phosphatidylinositol-specific phospholipase C (PI-PLC) and hemolytic activity (Johnson et al., 2004; Mengaud et al., 1991a).

While it is believed that the natural habitat of *Lm* consists of soil surface rich in decaying plant matter, it does not seem to belong to a strict environmental niche and has been isolated from many different sources including soil, vegetation, water courses (groundwater, sewage), silage, both farm and urban environments and animal feeds (Sauders et al., 2012; Thévenot et al., 2006). Food processing environments are another important site of *Lm* isolation. Food processing plants are critical for *Lm* spread since one single source of contaminated food products can shortly reach people all around the world (Thévenot et al., 2006). The presence of *Lm* in varied environments is due to its versatile physiology characterized by growth at temperature between 1°C and 45°C, at pH range of 4.4-9.6 and in presence of high salt concentration (10% NaCl). It can also survive but not grow under harsher conditions: high hydrostatic pressure, acidic pH as low as 2.5, bile (>0.3%) and bile acids (>5 mM) (Chaturongakul et al., 2008). This makes *Lm* particularly prone among foodborne pathogens to growth in well refrigerated foods with high salt content and low humidity and, thus, difficult to eliminate by some common food decontamination processes (Ibarra-Sanchez et al., 2017; Maertens de Noordhout et al., 2014). Whereas it cannot form spores, *Lm* high stress resistance and persistence is many times linked to its ability to form biofilms (Colagiorgi et al., 2017). Despite being classified as a ubiquitous saprophyte in the environment, the ability of *Lm* to adapt to both the life in the soil as well as in the cytosol of eukaryotic cells allows for a transition into a dangerous pathogen, capable of infecting a range of different cell types causing disease not only in humans but also in different animals including mammals, birds, cold-blooded vertebrates and arthropods (Cossart and Lebreton, 2014; Lecuit, 2007).

At the genotypic level, the genome of *Lm* and other *Listeria* species are generally similar. They consist of circular chromosomes with a low G+C content (average of 38%), sizes comprised between 2.7 and 3.0 Mb and contain about 2800 putative protein encoding genes (Hain et al., 2006). *Listeria* genomes also show a good amount of conservation regarding genetic content and organization since rearrangements and inversions of considerably large DNA fragments appear to be rare in the genus (Hain et al., 2006). However, some small genomic differences are clear and consistent between some species. Notably, one that defines the origin of pathogenicity of the genus is the 9 kb *Listeria* pathogenicity island 1 (LIPI-1) (Figure 1). The LIPI-1 segment comprises seven genes (*prfA, plcA, hly, mpl, actA, plcB, orfX*) which are some of the most crucial virulence factors needed for key steps of the *Lm* cell infection cycle and, thus, essential for intracellular parasitism (Prokop et al., 2017; Vázquez-Boland et al., 2001). The LIPI-1 element is present and functional almost exclusively in pathogenic strains of *Lm* and *L. ivanovii* (Chakraborty et al., 2000). Both the fact that *L. seeligeri* also displays a similar

virulence island that is rendered non-functional due to disruption of its autoregulatory loop and the fact that LIPI-1 identified in the three species lack any typical traces of mobility elements and show G+C content, codon usage and dinucleotide frequency consistent with the rest of the chromosome, contribute to the theory that the common ancestor of the *Listeria* species harbored this genetic element (Chakraborty et al., 2000; Vázquez-Boland et al., 2001).

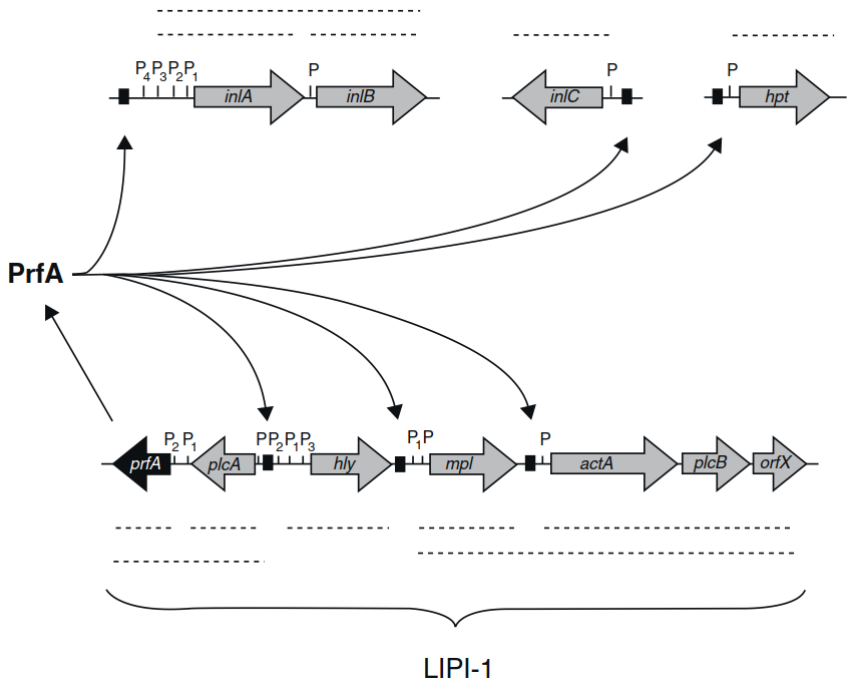

Figure 1 – PrfA core regulon. The PrfA regulon comprises *Listeria* pathogenicity island-1 (LIPI-1) encoding LLO (*hly* gene), ActA, PlcA, PlcB, Mpl and PrfA, plus three additional chromosomal loci: the *inlAB* operon and the *inlC* and *hpt* monocistrons. Genes pointing to the right are on the positive strand. PrfA boxes are indicated by black squares, known promoters and transcripts are indicated by 'P' and dotted lines respectively. (Adapted from de las Heras et al., 2011)

As a pathogenic bacteria *Lm* is also well adapted to the host environment. After ingestion by a susceptible human or animal *Lm* goes through a set of physiological changes which allows for survival and proliferation inside host cells. This transition is characterized by changes in gene expression, specially an increase of expression of genes that contribute to survival, replication and spread in host cells. In order to have such a dual lifestyle, *Lm* must be capable of detecting various signals and cues from both types of habitats in order to correctly adapt and prevail in such different environments. (Chaturongakul et al., 2008). One of the most important ways for bacteria to successfully adapt to changing stressful environmental conditions is through the expression of alternative σ factors. These factors are transcription initiation factors that consist of an essential subunit of the RNA polymerase holoenzyme and promote recognition of specific DNA by the enzyme. A single factor can regulate hundreds of genes

and the interplay between different alternative σ factors, thus, allows for a quick transcription reprograming upon stressful conditions (Chaturongakul et al., 2008; Kazmierczak et al., 2005). The genome of *Lm* encodes four σ factors, σ^B, σ^C, σ^H and σ^L (Glaser et al., 2001). Among these, σ^B has been shown to have a very important role in stress adaptation, both outside and inside the host, by controlling a regulon of more than 170 genes (Abram et al., 2008).

Listeria pathogenesis

Listeriosis

Lm is the etiological agent of the potentially fatal infection listeriosis. Despite the fact that clinical cases of listeriosis are still considered relatively rare (0.1-10 cases per million people, 0.1% of foodborne infections) and mostly confined to susceptible individuals, it has a high fatality rate of 20-30% (Swaminathan and Gerner-Smidt, 2007). Recent reports revealed a significant increase in listeriosis incidence since 2008 and in 2015 270 deaths were reported in Europe (Table 1) (EFSA Report, 2016). High risk groups include the immunocompromised, infants, the elderly and also pregnant women (Allerberger and Wagner, 2010; Vázquez-Boland et al., 2001). Healthy individuals infected by *Lm* might experience a milder and self-limiting gastroenteritis but individuals who suffer from the invasive form of listeriosis usually develop severe meningitis, encephalitis or even septicemia. Aside from the general symptoms showed by patients of invasive listeriosis like high fever, vomiting, headaches, nausea and meningeal irritation, a number of focal infections like pleuropulmonary, joint and skin infections, hepatic, splenic and brain abscesses, peritonitis, arthritis, among others have also been described (Allerberger and Wagner, 2010; Doganay, 2003).

Table 1 – Reported hospitalization and case fatality rates due to zoonoses in confirmed human cases in the EU, 2015. (Adapted from EFSA Report, 2016)

Disease	Number of confirmed[a]	Hospitalisation				Deaths			
	Human cases	Status available (%)	Number of reporting MS[b]	Reported hospitalised cases	Proportion hospitalised (%)	Outcome available (%)	Number of reporting MS[b]	Reported Deaths	Case Fatality (%)
Campylobacteriosis	229,213	27.0	17	19,302	31.2	73.7	16	59	0.03
Salmonellosis	94,625	34.0	16	12,353	38.4	55.6	16	126	0.24
Yersiniosis	7,202	23.9	14	530	30.9	59.8	14	0	0.0
STEC infections	5,901	39.4	14	853	36.3	56.2	15	8	0.24
Listeriosis	2,206	44.9	18	964	97.4	69.1	20	270	17.7
Tularaemia	1,079	14.9	9	89	55.6	15.6	10	0	0.0
Echinococcosis	872	20.5	13	107	59.8	23.5	13	1	0.49
Q fever	833	NA[c]	NA	NA	NA	47.7	12	3	0.36
Brucellosis	437	42.8	8	130	69.5	31.1	8	1	0.74
Trichinellosis	156	72.5	7	30	34.5	75.0	8	0	0.0
West Nile fever[a]	127	51.2	7	54	83.1	51.2	5	2	1.57
Rabies	0	NA[c]	NA	NA	NA	0.0	0	0	0.0

(a): Exception made for West Nile fever where the total number of cases was included.
(b): Not all countries observed cases for all diseases.
(c): NA-not applicable as the information is not collected for this disease.

Lm can be found in various types of food from raw to processed products. Notably deli meats, pâté and meat spreads, milk, cheeses and dairy products, smoked, cooked and ready-to-eat seafood are among the foods most related with clinical cases of *Lm* infection (Swaminathan and Gerner-Smidt, 2007). Since the infection dose for humans appears to be high, the robust physiology of *Lm* is crucial for its multiplication too occur in these products even when refrigerated or in other unfavorable conditions for bacterial growth (Morris Jr and Potter, 2013; Vázquez-Boland et al., 2001). In fact, *Lm* has been detected in food products as little as <1 CFU per g to very high numbers (>10^5 CFU per g) (Gombas et al., 2003; Wiedmann and Sauders, 2007). The *Lm* incubation time is believed to be around three weeks but cases due to a contaminated product have been reported as soon as a few days to as late as two months after exposure. The diagnosis of listeriosis is typically done by direct culturing on media plates from preferably sterile samples such as blood, cerebrospinal fluid, placenta but also gastric washes. Gram staining and microscopic analysis are not a preferable method for clinical specimens and the use of PCR is a more reliable option for quick detection (Allerberger and Wagner, 2010). The treatment of listeriosis depends greatly on antibiotic administration. For more than four decades the scene of *Lm* antibiotic resistance has remained fairly unchanged and the administration of a singular or combination of antibiotics is currently the most used treatment. While ampicillin and penicillin either alone or together with gentamicin are still the preferred options, the use of amoxicillin, trimethoprim–sulfamethoxazole, erythromycin and vancomycin are some favored alternatives, especially for patients with allergy to β-lactams or pregnant women (Conter et al., 2009; Heger et al., 1997; Hof, 2003). Once the pathogen is ingested, the main route of dissemination in the mammalian host is crossing the intestinal barrier (Figure 2).

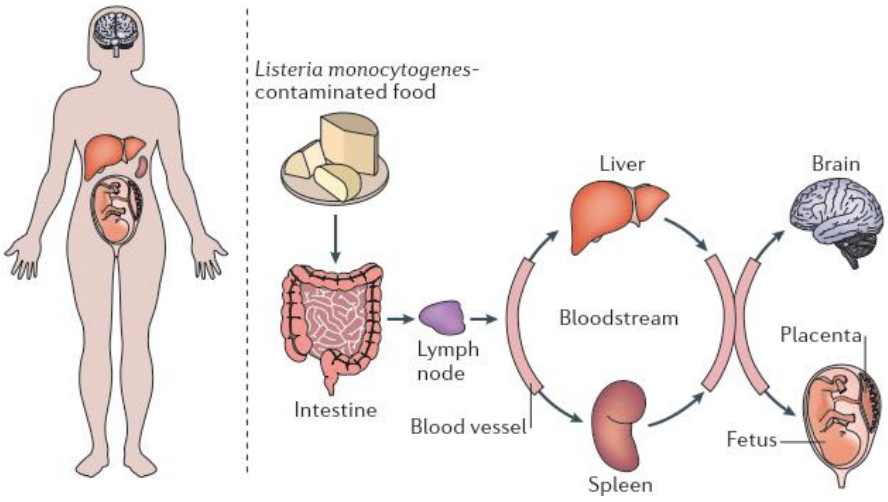

Figure 2 - Schematic of *Lm* infection of a human host. After ingestion of contaminated food, *Lm* can traverse the intestinal barrier and spread into the bloodstream through the lymph nodes to disseminate to target tissues, such as the liver and spleen. In immunocompromised individuals, *Lm* can cross the blood–brain barrier or fetoplacental barrier and cause potentially fatal meningitis, sepsis, premature birth or abortion. (Adapted from Radoshevich and Cossart, 2017)

Before reaching the intestine, however, *Lm* journeys through the digestive tract, a hostile environment where it faces several aggressions (Gahan and Hill, 2005). To cross the intestinal barrier *Lm* has to invade the intestinal epithelium cells (predominantly enterocytes) and translocate into the lamina propria (Figure 3). When crossing this barrier *Lm* can infect and spread across the epithelial cells or stay in the internalization vacuole of goblet cells and quickly transocytose through the cell (Nikitas et al., 2011). *Lm* is then carried by the lymph and the bloodstream to other organs, the liver and the spleen being the preferential organs for bacteria multiplication (Toledo-Arana et al., 2009). Resident liver and splenic macrophages will uptake and eliminate the bacteria in order to contain the infection but the adaptation to the intracellular environment will allow some *Lm* to survive and multiply inside these cells. Free bacteria invade non-phagocytic cells in these organs further spreading the infection and, if the immune response does not successfully contain *Lm* proliferation, the bacteria can circulate uncontrolled in the bloodstream causing bacteremia. This requires an effective T cell response which provides a faster elimination of the bacteria and provides protection against a reinfection (Zenewicz and Shen, 2007). This relevance of cell mediated immunity in containing *Lm* infection reflects how the state of the immune status of an individual defines susceptibility to listeriosis.

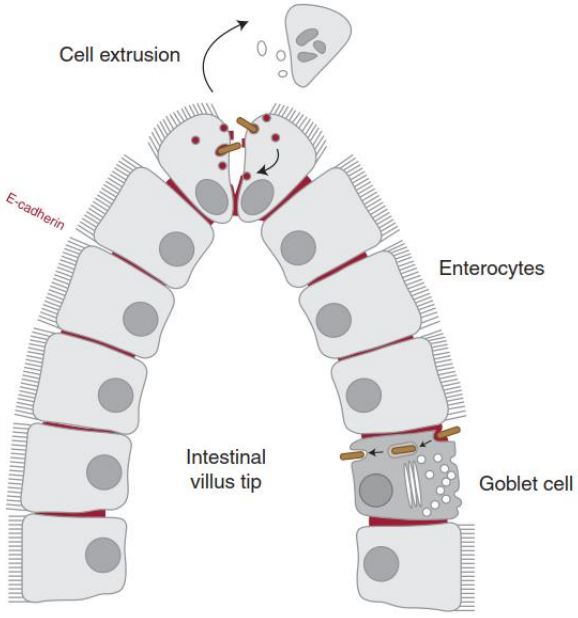

Figure 3 - Traversal of the intestinal barrier by the InlA-E-cadherin interaction. The first contact between *Lm* and host target cells takes place at the intestinal barrier level. E-cadherin, which is normally present at the basolateral face of enterocytes and therefore not exposed to the intestinal lumen, can be accessible to the *Lm* invasion protein InlA at sites of apoptotic cell extrusion at the villus tip. However, the main route for bacterial translocation across the intestinal barrier is through mucus-secreting goblet cells via a rapid transcytosis mechanism that does not require intracellular bacterial escape from its internalizing vacuole. (Adapted from Pizarro-Cerdá et al., 2012)

Because *Lm* is able to cross two other major physiological barriers, the blood-brain and the fetoplacental barriers, circulation of bacteria in the blood can lead to progression of the infection to other organs and development of aggravating symptoms (Radoshevich and Cossart, 2017). When crossing the blood-brain barrier *Lm* can infect the nervous system and the brain, resulting in meningoencephalitis and potentially resulting in severe sequelae (Disson and Lecuit, 2012). During pregnancy *Lm* can infect the placenta and reach the unborn child which can lead to serious complications to the newborn or interruption of pregnancy (Bakardjiev et al., 2006). Whereas the occurrence of clinical cases of *Lm* infection is mostly of a sporadic nature and with higher incidence in well developed countries, the pathogen is so widespread that both environmental isolates as well as cases of both human and animal listeriosis have been reported in all continents, with the exception of Antarctica (Orsi and Wiedmann, 2016). Despite this, outbreaks of large dimension and with serious human casualties have also been reported on many occasions. Large listeriosis outbreaks date back to as early as 1966 where at least 279 detected cases due to *Lm* infection where reported in the Halle region in Germany during that year (Wagner and McLauchin, 2008).

In 2011 a multistate outbreak with origin in contaminated cantaloupes occurred in the USA with a toll of 147 infected people which tragically resulted in 33 deaths (https://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html). In the current year of 2017 a large outbreak is being monitored in South Africa since January. As of November, the latest official report indicates a total of 557 laboratory-confirmed listeriosis cases of which 36 were fatal (http://www.health.gov.za/index.php/component/phocadownload/category/434). The outbreak is still currently ongoing and the source of infection has not been identified yet, although is it thought to be a widely available food product. In Portugal, only since 2014 has listeriosis become a notifiable disease. However, the country still lacks an appropriate active surveillance program. A retrospective study detected the occurrence of an outbreak between 2009 and 2012 confined mostly to the center region of Portugal, with cheese as the probable source of infection. The outbreak was characterized by a long period of 16 months from start to its detection and a high mortality of 11 casualties of a total of 30 cases (Magalhães et al., 2015). Among the identified *Lm* serotypes, three of them alone (1/2a, 1/2b and 4b) account for the majority of all clinical isolates (Farber and Losos, 1988). Although *Lm* and *L. ivanovii* are commonly regarded as the only pathogenic species and *Lm* as the only capable of causing human listeriosis, on rare occasions *L. ivanovii* has been isolated from patients presenting listeriosis symptoms (Beye et al., 2016; Cummins et al., 1994; Elischerová et al., 1990; Guillet et al., 2010; Lessing et al., 1994; Snapir et al., 2006) and even *L. innocua* has been associated with a fatal case of listeriosis (Perrin et al., 2003). The economic burden of *Lm* is also a relevant concern since it has become one of most costly foodborne pathogens not only in treatment and prevention but

also in nonmedical economic losses (Ibarra-Sanchez et al., 2017; Ivanek et al., 2004; Scharff, 2012).

Cell infection cycle

One of the main features of *Lm* that allows it to cross several physiological barriers is its unusual ability to invade and multiply into both non-phagocytic and phagocytic cells like professional macrophages (Cossart et al., 2003). The cell infection cycle of *Lm* can be separated in six main events: adhesion, invasion, vacuole escape, multiplication, actin-based motility and cell-to-cell spread (Figure 4).

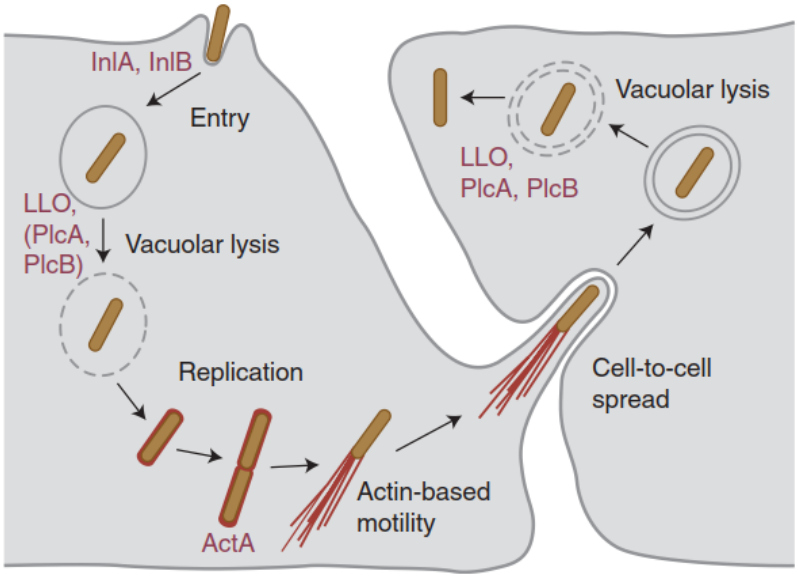

Figure 4 – Schematic representation of the intracellular cell cycle of *Lm*. (Adapted from Pizarro-Cerdá et al., 2012)

During each of these steps different bacterial factors play specific strategic roles, favoring survival and infection success. The two initial steps occur almost simultaneously and consist on the physical contact between the pathogen and the host cell, followed by bacterial internalization. *Lm* deploys two classes of surface proteins called adhesins and internalins that establish a stable interaction between the bacterial and host cell membranes and rapidly promote the internalization of *Lm* into non-phagocytic cells. Internalization into epithelial cells is an active process promoted by the bacteria (in the case of phagocytes it is the host cell that actively engulfs the bacteria) where interaction with specific host cell receptors triggers signaling cascades and recruitment of cytoskeleton components which, in a complex and finely

controlled process of a zipper-like mechanism of cytoskeleton reshaping and membrane extension (Figure 5), leads to the engulfment of *Lm* in a vacuole into the cytosol (Pizarro-Cerdá et al., 2012). The cell eliminates bacteria-containing vacuoles by conversion of the vacuole into a phagolysosome. To avoid killing, *Lm* deploys a defense mechanism of escape characterized mainly through secretion of a pore-forming cytolytic toxin that causes vacuolar membrane destabilization and eventual rupture, releasing the bacteria free in the cytosol. Shortly after, cytosolic *Lm* exploits the host cell own nutritious pool to uptake required nutrients in order to divide intracellularly. Since *Lm* lacks flagelar-based motility at the physiological temperature, it deploys an alternative system of actin-based motility for navigation inside the host cell. Bacteria express specialized proteins that hijack the host own polymerization machinery to nucleate actin, leading to the formation of a structure denominated as an actin tail or actin comet (Kocks et al., 1992). This creates the propulsion force necessary for bacterial intracellular movement. In the last step of the infection cycle, cell-to-cell spread, motile bacteria make contact with the inner side of the cell membrane creating a pseudopod-like protrusion that is phagocytized by a neighbor cell in the tissue, creating a double membrane vacuole. Bacteria escape from this secondary vacuole in a similar mechanism displayed for primary vacuoles and reinitiates the infection cycle spreading across the infected tissues while keeping protection from the immune surveillance extracellularly (Cossart and Toledo-Arana, 2008; Cossart et al., 2003). Recent findings have revealed the ability of *Lm* to perform a switch between this active motile lifestyle and a persistent vacuolar lifestyle. The switch was characterized to happen after days of colonizing hepatocytes or trophoblasts and initiates as the bacteria turns off its actin-based motility. *Lm* then becomes trapped inside lysosome-like *Listeria*-Containing Vacuoles (LisCVs) where it can stay for long periods of time in a persistent non-replicative form. *Lm* then switches back to its active form and is able to reinitiate the cell infection process. This switch is thought to favor *Lm* dissemination by promoting asymptomatic carriage, increasing incubation times and protection during antibiotic treatment (Kortebi et al., 2017).

Arsenal of virulence

Adaptation of a pathogen to the host environment and, in the case of *Lm,* the ability to invade and multiply inside host cells requires specialized genetic determinants, encoded by the genome of pathogenic *Listeria* strains, called virulence factors (Thomas et al., 2014). Virulence genes and their products are considered those that contribute for any stage of the infection progress or transmission of disease (Kazmierczak et al., 2005). Today, the list of identified and

characterized *Lm* virulence factors is too long to review here in detail. The following are some of the major virulence factors of *Lm* that play crucial roles throughout the cell infection cycle.

Internalins A (InlA) and B (InlB) play a major role at the cell internalization step by binding to specific surface receptors that trigger the internalization process (Figure 5) (Dramsi et al., 1995; Gaillard et al., 1991). These two major *Lm* invasins belong the internalin protein family which is characterized by a typical structure composed by a signal peptide, a leucine-rich repeat (LRR) domain, a conserved inter-repeat (IR) domain and typically an anchoring domain. The LRR domain is characterized by leucine-rich 20-22 amino acids tandem repeats, involved in ligand-receptor binding and other protein-protein interactions and adhesion (Cabanes et al., 2002). The LRR and the IR regions are needed and sufficient for *Lm* internalization (Lecuit et al., 1997). At the C-terminal InlA contains a signature sequence motif denominated LPXTG which provides a covalent and stable anchoring to the cell wall mediated by the sortase A enzyme (Lebrun et al., 1996). InlB a contains a GW motif instead, which is also required and sufficient to anchor the internalin to the bacterial cell wall, through non-covalent bonds with lipoteichoic acids (LTAs) or peptidoglycan (Braun et al., 1997; Milohanic et al., 2001; Percy et al., 2016). The receptors at the host cell membrane for these internalins are the cell-cell adhesion mediating transmembrane glycoprotein E-catherin in the case of InlA (Mengaud et al., 1996) and c-Met is the major InlB receptor, although other receptors like gC1qR and glycosaminoglycans have been identified (Figure 5) (Braun et al., 2000; Camejo et al., 2011; Shen et al., 2000). Since the interaction of InlA and InlB and their receptors is species specific, the tropism of each internalin determines not only what type of cell *Lm* can invade but also which hosts are susceptible to infection (Figure 6) (Camejo et al., 2011). Whereas InlB mediates entry into several cell types (Vero, HeLa, CHO, Hep2), InlA mediates entry of cells of epithelial origin (Caco-2) (Lecuit et al., 1997). Internalins not only are sufficient for *Lm* cell internalization, they also confer the non-pathogenic *L. innocua* an invasive phenotype (Braun et al., 1998; Gaillard et al., 1991). The invasion of the human intestinal epithelium is greatly dependent on InlA and its affinity to human E-cadherin but due to a residue substitution it is not able to bind to the mouse homologue (Camejo et al., 2011; Lecuit et al., 1999). The role of InlA and InlB on the fetal-plancental barrier is not fully clear but evidence suggests that the cooperation of InlA and InlB is important for the invasion of the placenta (Disson et al., 2008). Unlike most of the major virulence factors implicated in the cell infection cycle, InlA and InlB are not contained in the LIPI-1 element but rather in a distinct locus of the chromosome (Vázquez-Boland et al., 2001). The cholesterol-dependent cytolysin (CDC) listeriolysin O (LLO) is a secreted pore-forming toxin encoded by the *hly* gene and is one of the most important and versatile *Lm* virulence factors. For having multiple roles promoting infection and affecting the host cell from the different *Lm* locations throughout infection (extracellular medium, cytosol and

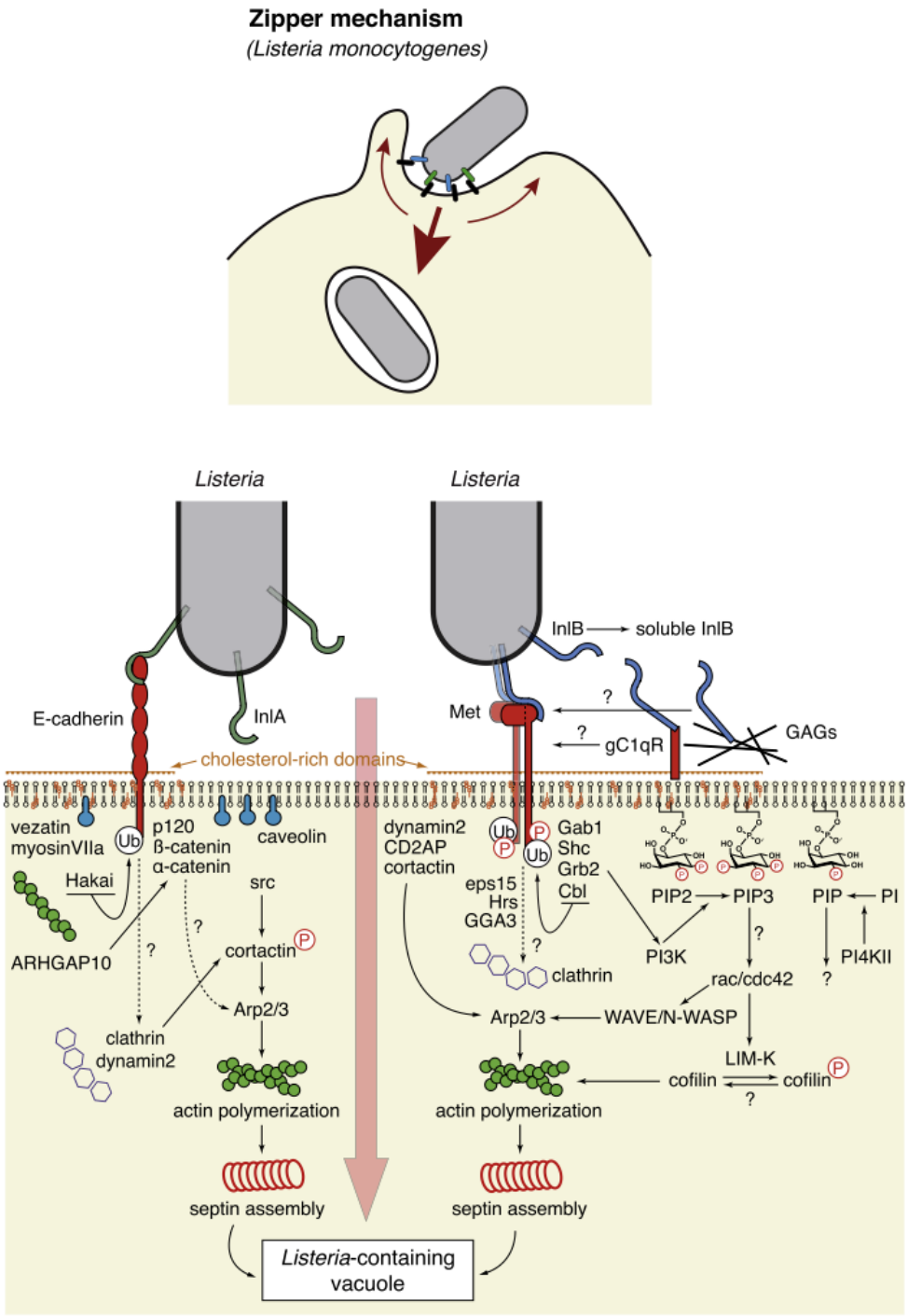

Figure 5 - Schematic representation of internalization of *Lm* via a "zipper" mechanism. (Adapted from Ribet and Cossart, 2015)

vacuole), LLO became commonly referred to as the *Lm* army-knife (Hamon et al., 2012; Osborne and Brumell, 2017). From the extracellular medium LLO is implicated in: efficient internalization into HepG2 cells, in a dynamin and actin-dependent mechanism upon pore-formation (Vadia et al., 2011); induction of several host cell signaling pathways such as multiple kinases pathways (Tang et al., 1998; Weiglein et al., 1997), phosphatidylinositol metabolism, nuclear translocation of NF-kB (Kayal et al., 1999) and secretion of the cytokines IL-6, IL-8,

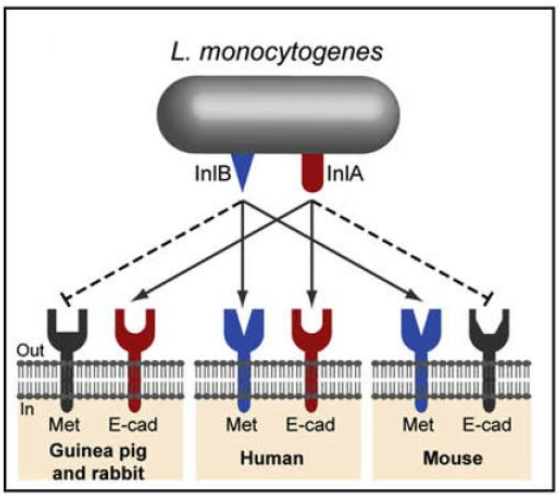

Figure 6 - Species specificities of InlA and InlB. (Adapted from Cossart and Toledo-Arana, 2008)

GM-CSF and IL-1a (Osborne and Brumell, 2017); induction of apoptosis during infection which has been demonstrated to increase susceptibility to infection by promotion of IL-10 expression (Carrero and Unanue, 2012; Rogers et al., 1996). From inside the vacuole LLO also plays several important roles such as reducing the production of ROS by the host, formation of spacious *Lm*-containing phagosomes where *Lm* can grow inside and promote chronic type infection (Bhardwaj et al., 1998; Birmingham et al., 2008) and the induction of autophagy, although the relationship between this phenomenon and *Lm* infection is not fully understood (Osborne and Brumell, 2017; Zhao et al., 2008). However, the hallmark role of LLO is the disruption of the primary and secondary vacuoles to avoid intracellular killing and to gain access to the cytosol. *Lm* secretes LLO monomers which oligomerize at the vacuole membrane, forming pores which lead to vacuolar disruption. LLO appears to be the only known CDC toxin to have pH dependent activity which is favored at the slightly acidic pH of the vacuole and together with its controlled expression allows for a safe pore formation, which could otherwise disrupt other organelles or even the cell membrane, leading to exposition of *Lm* to the extracellular immune surveillance (Glomski et al., 2003; Hamon et al., 2012). From the cytosol of the host cell LLO has the following additional functions: the poorly understood mechanism of calcium-dependent mitochondrial network fragmentation (Stavru et al., 2011); the pore-induced host protein degradation and interference with protein SUMOlyation (Hamon et al., 2012; Ribet et al., 2010); activation of the inflammasome (Eitel et al., 2011) and damage to the endoplasmic reticulum and induction of unfolded protein response although these two particular phenomena do not seem to favor *Lm* infection (Gekara et al., 2007; Pillich et al., 2015). Mutants for the LLO-encoding gene *hly* were shown to be restricted to the vacuole intracellularly and have an avirulent-type phenotype *in vivo* (Osborne and Brumell, 2017).

Other important virulence factors also mediate the disruption of the primary and secondary vacuoles. Namely, the phosphatidylinositol-specific phospholipase C (PI-PLC) and the broad-range phospholipase C (PC-PLC) work together with LLO in the membranar disruption, the latter whose maturation and function is mediated by the zinc metalloprotease Mpl (Bitar et al., 2008; Domann et al., 1991; Geoffroy et al., 1991; Leimeister-Wächter et al., 1991; Poussin et al., 2009; Poyart et al., 1993; Yeung et al., 2005).

During the intracellular multiplication step of the *Lm* infection cycle, bacteria express another important virulence factor, the hexose phosphate transporter Hpt. Hpt mediates the uptake of hexose phosphates that are available in the host cytosol and that bacteria use as a carbon source to promote intracellular multiplication (Chico-Calero et al., 2002). In macrophages, the secreted virulence factor OrfX promotes intracellular survival and multiplication (Prokop et al., 2017). OrfX, is encoded by the last gene of LIPI-1 and was, until very recently, the only factor of this cluster to be of unknown functions. This novel virulence factor has now been described to contribute to intracellular survival and successful infection of macrophages by targeting and reducing the levels of a nuclear host regulatory protein (RybP) and by dampening the macrophage oxidative stress (Prokop et al., 2017).

Another major virulence factor is ActA, a surface and polarized protein that in addition to roles in cell adhesion and invasion plays a crucial function in *Lm* intracellular mobility and cell-to-cell spreading (Kocks et al., 1992; Pillich et al., 2016). ActA locates to one of the bacterium poles and promotes the polymerization of actin filaments by mimicking some host actin nucleating factors (like the WASP family of proteins) and by recruitment and activation of the Arp2/3 complex another important host actin nucleator (Boujemaa-Paterski et al., 2001; Campellone and Welch, 2010; Welch et al., 1997). Actin-based mobility is absolutely dependent on ActA which without any other bacterial factors is sufficient for actin tail formation but is dependent on the action of the Ena/VASP family of host proteins to modulate bacterial speed and directionality (Auerbuch et al., 2003; Cameron et al., 1999; Skoble et al., 2001).

Virulence regulation

Regulation of expression of virulence genes in bacteria

Bacterial pathogens are equipped with a specific set of virulence factors, the virulome, which grants them adaptability to the host environment. The virulome can vary from a few hundreds to more than a thousand virulence factors and these are not constantly expressed nor expressed at the same levels (Thomas et al., 2014). In fact, constant or uncontrolled expression of such factors can become detrimental or energetically wasteful for the bacteria

(McKenney and Kendall, 2016; Vasanthakrishnan et al., 2015). Therefore, when a pathogen changes environments, notably when moving from their saprophytic habitat to their target host, they undergo a massive transcriptional shift as a response to various external signals (Camejo et al., 2009; Lam et al., 2015; Thomas et al., 2014; Toledo-Arana et al., 2009). Transcriptional changes are mainly mediated by genome encoded elements of the transcriptional regulatory machinery: transcription factors, σ factors, anti-terminator proteins and cis- and trans-acting non-coding RNAs. Transcription factors in particular are abundant, diverse, can sense various stimuli, be modulated and repress or promote gene expression (Figure 7) (Lloyd et al., 2001; Rodionov, 2007). Transcription factors responsible for controlling the expression of virulence factors are denominated virulence regulators. Whereas the spotlight of virulence regulation can be attributed to a singular virulence regulator (as is the case of *Lm*, described below) or a virulence regulatory system (like the Agr system of *Staphylococcus aureus*) that have a large set of virulence factors under control and exert a great influence on virulence, this is an over simplistic point of view which neglects a much larger, intricate and coordinated network of multiple virulence regulators (Vakulskas et al., 2015). Bacteria can sense different forms of environmental cues such as sensing of chemical molecules, changes in temperature and even changes or physical damage to the membrane (Flores-Kim and Darwin, 2014; Lam et al., 2015). Responses to one or more simultaneous factors can result in fine tuning of limited specific virulence genes or translate into a global-like change of transcription. It is well-established that virulence regulation occurs through mechanisms of signal transduction by two- or three-component systems, quorum sensing systems and ECF sigma factor-dependent systems. More recently, growing evidence have linked eukaryotic-like serine-threonine kinase/phosphatase-dependent (eSTK/eSTP) systems as novel ways of bacterial virulence regulation (Thomas et al., 2014).

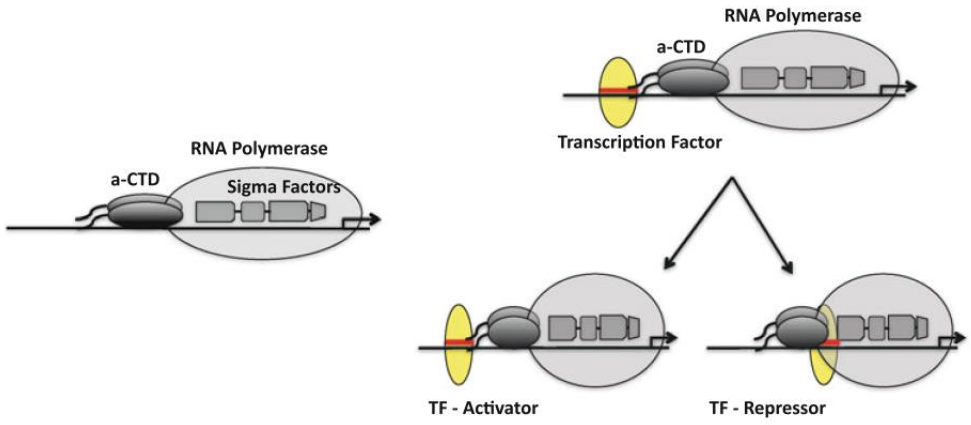

Figure 7 – Classical transcription factor regulation. Transcription factors (TF) interfere with RNA polymerase holoenzyme and activate or repress transcription depending on their binding site relative to the transcriptional start site. (Adapted from Sai et al., 2011)

Virulence regulation in *Lm*

Regulation of virulence genes in *Lm* is not a simple process. The positive regulatory factor A (PrfA) plays a central role as the positive regulator of the major virulence factors involved in the *Lm* infection cycle (Freitag et al., 2009). Often regarded as the master commander of *Lm* virulence, PrfA is a 27 kDa Crp/Fnr family transcription factor that enhances the transcription of target genes by recognition and binding to a palindromic DNA sequence in their promoter region known as PrfA-box (Freitag, 2006; Körner et al., 2003). Expression and proteomic analyzes have suggested that whereas the core regulon of PrfA is composed of twelve directly upregulated genes, the complete regulon comprises 145 other genes that are also putatively under its control (Milohanic et al., 2003; Monzo et al., 2007). These include other virulence factors outside the LIPI-1 island like the bile salt hydrolase Bsh and a bile exclusion system BilE which contribute to bile resistance and promote survival in the gallbladder and intestine (Begley et al., 2005; Dussurget et al., 2002; Gahan and Hill, 2005; Hardy et al., 2004; Sleator et al., 2005) and also the already described hexose transporter Hpt (Chico-Calero et al., 2002). Absence or malfunction of PrfA renders *Lm* unable to replicate intracellularly and spread across cells and results in a striking reduction in virulence in the mouse model (Freitag et al., 1993; Mengaud et al., 1991b). To ensure an appropriate control of virulence, the regulation of PrfA itself is a complex mechanism performed at different levels. At the transcriptional level PrfA is controlled at three promoters all controlled by PrfA itself, one positively while the other two negatively, which allows for distinct levels of PrfA activation (Vasil and Darwin, 2013). PrfA is also regulated at the post-transcriptional level by a trans-acting riboswitch (Loh et al., 2009) and a thermosensor switch that exists within the *prfA* mRNA 5' UTR (Figure 8). At temperatures up to 30°C the riboswitch forms a stem-loop structure that masks the ribosome binding site, preventing translation initiation (Johansson et al., 2002). At the physiologic temperature of 37°C this structure becomes unstable, exposes the ribosome binding site and allows for protein production. Interestingly, only one of the promoters contains this riboswitch suggesting the other two allow for some level of PrfA-dependent gene expression at environmental temperatures. PrfA is also controlled at the post-translational level. Notably, both host and *Lm* produced glutathione bind PrfA allosterically and this is necessary for its activation (Reniere et al., 2015). It is likely that *Lm* uses glutathione concentration to mediate its saprophyte-pathogen transcriptional switch. Additional evidence of post-translational regulation include reports of downregulation of PrfA-dependent genes when carbon sources like glucose or cellobiose are present, while PrfA levels are not significantly changed (Milenbachs et al., 1997) and an *Lm* strain encoding a PrfA protein mutated for the putative cofactor binding factor that showed higher affinity to the *hly* promoter (Eiting et al., 2005). Nonetheless, the regulatory

scene of virulence of *Lm* strays far from the simplicity of a single regulator and several other regulators with roles in virulence have been described.

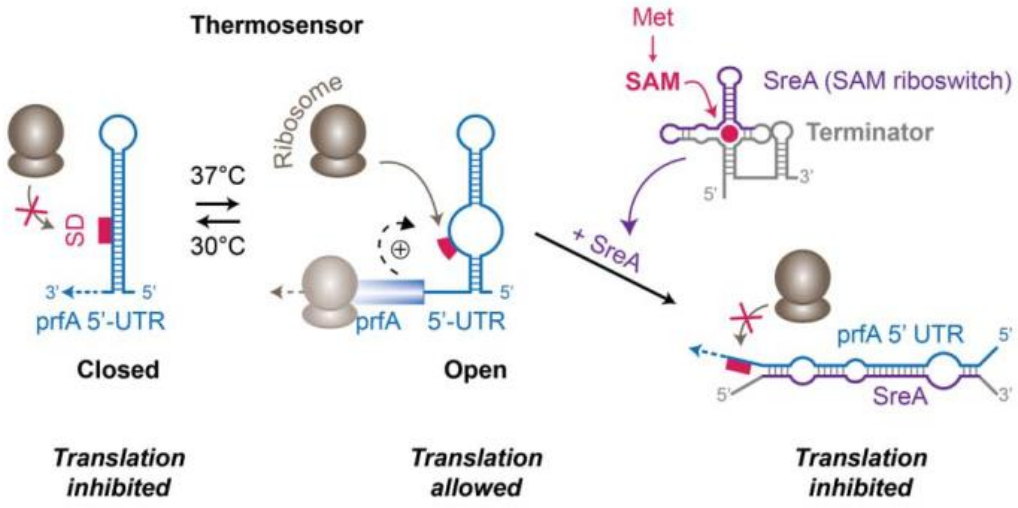

Figure 8 - PrfA control mechanisms. The Shine-Dalgarno (SD) sequence of *prfA* mRNA is masked from ribosomes by a closed stem-loop structure. At 37°C, a change in the conformation of the 5'-UTR liberates the SD and allows translation initiation. Binding of ribosomes to the SD is further stabilized by the 20 first codons of the ORF. The SreA sRNA, which is the product of a S-adenosylmethionine (SAM) riboswitch, can also base-pair with prfA 5'-UTR and block access of ribosomes to the SD sequence. (Adapted from Lebreton and Cossart, 2016)

VirR of the VirR/VirS two component-system of *Lm* is another characterized virulence regulator. VirR positively controls a set of genes among which some with relevant roles in *Lm* virulence like the *dlt* operon, related to the cell wall LTA D-alanylation, and *mprF* which confers resistance to host defensins by phospholipid lysinylation (Kang et al., 2015; Mandin et al., 2005; Thedieck et al., 2006). Indeed, both *virR* and most of its regulon were upregulated in mice spleen during infection and in infected macrophages, further suggesting a relevant role during infection (Camejo et al., 2009; Chatterjee et al., 2006). Like PrfA, VirR also recognizes and binds a specific DNA sequence in the promoter region of the genes it directly regulates (Mandin et al., 2005).

Regulation of flagella-dependent mobility components is a complex mechanism of regulation of an important feature of *Lm* needed for adaptation and virulence. Flagella have been shown to play a role in the invasion of host cells (Bigot et al., 2005; Dons et al., 2004; Hayashi et al., 2001; O'Neil and Marquis, 2006) and flagellar proteins also play immunogenic roles and trigger an immune response (Hayashi et al., 2001; Way et al., 2004). Flagellar gene expression is known to be temperature sensitive and *Lm* specifically downregulates flagellar gene expression at higher temperatures (close to 37°C) to avoid immune recognition but is flagellated and motile at lower temperatures up to 30°C. Regulation of flagella is complex and

several regulators are known to be involved. MogR is a transcription regulator that appears to function as the central flagella repressor at higher temperatures, repressing expression of the central flagella subunit FlaA and other flagella-related genes (Figure 9). At low temperatures the orphan response regulator DegU promotes the expression of another regulator GmaR that in turn inhibits MogR, allowing flagella expression (Gründling et al., 2004; Kamp and Higgins, 2009; Shen and Higgins, 2006).

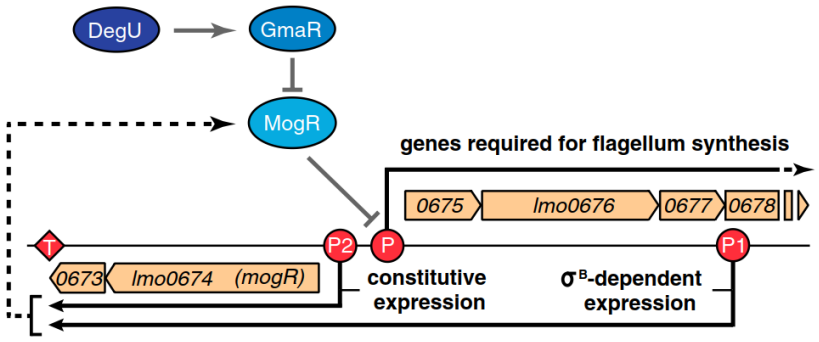

Figure 9 - Regulation of flagella expression. (Adapted from Cossart, 2011)

The CtsR regulator was identified in *Lm* as a class III heat shock gene negative regulator and was mainly associated to stress response but has since then been linked to virulence (Nair et al., 2000). A mutant for a glycine residue in the CtsR glycine repeat regulatory region was shown to be attenuated in virulence in the mouse model (Karatzas et al., 2003) and the ClpB factor which is under direct control of CtsR, was shown to be required for full virulence *in vivo* in a general stress response-independent manner (Chastanet et al., 2004).

CodY is a known sensor of branch-chained amino acids (BCAAs) and a global-regulator of metabolic and virulence genes in Gram-positive bacteria, mostly active under rich growth conditions (Sonenshein, 2005). In *Lm* CodY has been shown to have a very versatile role by activating and repressing several genes under both rich and minimal growth conditions. Notably, under low availability of BCAAs CodY modulates virulence by activating the expression of *prfA*, promoting the expression of PrfA-dependent virulence factors (Lobel et al., 2012, 2015). CodY is also capable of directly regulating the expression of several genes important for virulence, stress response and motility (e.g. *actA, sigB, fla* genes, etc.) (Lobel and Herskovits, 2016).

HrcA is a repressor of class I stress response genes that positively and negatively regulates the expression of several genes in *Lm* (Hu et al., 2007). HrcA has been shown to play a role in biofilm formation, heat and acid shock resistance and evidence suggest a possible

implication in cell invasion (Hu et al., 2007; Ivy et al., 2012; van der Veen and Abee, 2010). HrcA also appears to co-regulate several genes with other notable regulators such as σ^B, σ^H and CtsR (Chaturongakul et al., 2011; Hu et al., 2007).

Once considered rare in bacteria, the class of short noncoding RNAs (sRNA) is now an established and relevant component of regulatory networks (Storz et al., 2011; Waters and Storz, 2009). Either encoded as antisense from the opposite strand of their target or trans-encoded from a distant location, sRNAs usually control gene transcription by imperfect base pairing with their target mRNAs, most commonly by directly blocking the ribosome binding site, interfering with ribosome binding at a farther site, increase ribosome binding by preventing secondary mRNA structure formation and also by altering mRNA stability (Storz et al., 2011). Whereas the trio LhrA, LhrB and LhrC were the first sRNA to be identified in *Lm*, the use of bioinformatics and the fast evolving array and sequencing platforms allowed the discovery of over 100 new elements of this class (Christiansen et al., 2006; Mandin et al., 2007; Mohamed et al., 2011; Oliver et al., 2009; Toledo-Arana et al., 2009).

Evidence suggests that LhrA has a very wide regulon since an *lhrA* mutant had almost 300 genes differently regulated when grown *in vitro* (Nielsen et al., 2011). Notably, LhrA directly regulates the chitinase ChiA by pairing to its mRNA ribosome binding site and inhibiting translation and this regulation has been shown to link LhrA and *Lm* virulence (Nielsen et al., 2011). ChiA performs the enzymatic hydrolysis of chitin and plays a metabolic role but has also been proposed as virulence factor, as a host immune suppressor. A mutant for *chiA* showed an attenuated virulence phenotype in the mouse model and was shown to induce higher expression of the inducible nitric oxide synthase (iNOS), a component of the immune response (Chaudhuri et al., 2013).

LhrC was initially discovered as five copy sRNA found to be highly expressed in *Lm* under infection conditions, namely under cell envelope stress, but was recently shown to have two other copies, becoming the largest sRNA family of *Lm* (Mollerup et al., 2016). LhrC plays a role in virulence by contribution to macrophage-like cell infection and by direct regulation of several virulence factors including the cell wall associated adhesin LapB, the oligopeptide binding protein OppA and the CD4+ T cell-stimulating antigen TcsA (Sievers et al., 2014, 2015). The expression of all copies of LhrC was, in turn, found to be highly dependent on the two-component system LisRK (Sievers et al., 2014).

The identification and characterization of these elements keeps increasing (Radoshevich and Cossart, 2017). Recently, an interplay of the regulatory sRNA Rli31 and the Gram-positive conserved but newly characterized RNA binding protein SpoVG has been described to have roles in *Lm* virulence, including regulation of genes like *pgdA* and *pbpX* with significant effects

on lysozyme resistance (Burke and Portnoya, 2016; Burke et al., 2014). Another recent study revealed the mode of action of the regulatory sRNA Rli55 and its impact in *Lm* virulence. Here, Rli55 controls the expression of ethanolamine utilization *eut* genes by sequestering of the two-component system regulator EutV in an RNA-protein interaction. Rli55 itself is, in turn, regulated by a vitamin B_{12} dependent riboswitch, adding another regulatory layer (Mellin et al., 2014).

Hfq is an RNA-binding chaperone highly conserved in prokaryotes. While the precise mechanism of Hfq action is still under debate, it binds and stabilizes sRNAs, mediates RNA-RNA interactions and mediates regulation (Christiansen et al., 2004). mRNA binding by LhrA has been demonstrated to be mediated by Hfq in *Lm* (Nielsen et al., 2009).

Although it has a large regulon that is mainly related to stress response and adaptation, the σ^B factor also plays a role in virulence regulation. A deletion mutant for σ^B is impaired both in the invasion of eukaryotic cells and in virulence in the mouse model, suggesting an important role in virulence (Kim and Boor, 2004; Wiedmann et al., 1998). Among the genes under the control of σ^B are the following virulence associated genes: the bile salt hydrolase *bsh* and the bile exclusion system *bilE* (Dussurget et al., 2002; Sleator et al., 2005), the RNA-binding regulatory protein-coding *hfq* (Christiansen et al., 2004), the carnitine osmotransporter system *opuC* (Fraser et al., 2003; Wemekamp-Kamphuis et al., 2002) and internalins *inlA* and *inlB* (Kim and Boor, 2004; McGann et al., 2007; Sue et al., 2004). Additionally, σ^B also regulates other virulence regulators. Importantly, not only there is an overlap between PrfA and σ^B regulons, σ^B also appears to have a transcription control over PrfA itself which further implicates this factor in *Lm* virulence regulation (Milohanic et al., 2003; Nadon et al., 2002; Schwab et al., 2005).

GntR family of transcription regulators

Discovery and general features

Transcription factors represent a major way of gene expression regulation in bacteria and typically represent a good portion of the genome. It is believed that transcription factors account for approximately 10% of the bacterial genomes (Rodionov, 2007). Regulation is based on DNA-protein interaction and mechanisms for understanding and predicting these interactions have been a hot topic in the domains of computational biology and molecular biology (Suvorova et al., 2015). Although bacterial transcription factors represent an abundant and diverse class of proteins, our knowledge on transcription factor structure and function is still limited and a very low percentage of structural data for all transcription factors described

in the literature is available (Ofran et al., 2007). Several groups have been established based mostly on conserved motifs and modes of DNA binding like the helix-turn-helix (HTH), β-sheet DNA-binding, homeaodomain, leucine zipper and zinc finger domains (Harrison, 1991; Pabo and Sauer, 1992). One of the most recognizable family of transcription factors is the GntR family. The name was attributed after the discovery of its first element over two decades ago in 1991, the gluconate-operon repressor of *Bacillus subtilis* (Fujita et al., 1986; Haydon and Guest, 1991). Since then, this protein family as greatly expanded, with members identified across diverse bacteria, playing roles in various biological processes and even became a popular reference for studies in DNA-protein interaction. GntR regulators are mostly recognized for roles in primary metabolism through regulation of diverse metabolic pathways (e.g. fatty acid, amino acid, organic acid and carbon metabolism, etc.) (Hoskisson and Rigali, 2009).

GntR transcription factors share a similar overall structure composed of a conserved DNA-binding site at the protein N-terminal and an effector-binding or oligomerization (E-O) domain at the C-terminal (Suvorova et al., 2015). The DNA-binding domain of the members of this family shows high level of conservation and is characterized by a β-sheet core and three α-helices (typically as a α1α2α3β1β2 two-dimensional structure). The HTH domain is characterized by α-helix, a turn (the connecting loop) and a second α-helix, known as the recognition helix that fits into the major groove of DNA. Interestingly it is the overall topology and structure that is so typical across the family as, in fact, the amino acid identity conservation of this domain is only approximately 25% (König et al., 2009; Rigali et al., 2002).The E-O domain is located at the C-terminal and is not implicated in DNA binding but rather in the protein oligomerization and/or ligand binding. While this domain confers no DNA specificity it can mediate conformational changes and steric constraints on the DNA-binding domain, destabilizing or enhancing DNA-binding, modulating gene regulation (Rigali et al., 2004).

The genome of *Lm* EGD-e is predicted to encode between 19 and 24 putative GntR proteins, as the largest family of regulators in this strain (Buchrieser et al., 2003; Glaser et al., 2001). However, to our knowledge only one attempt was made to characterize a GntR regulator of *Lm*. LbrA was identified in *Lm* Scott A strain as a GntR-family regulator that is implicated in biofilm formation and potentially regulates more than 300 genes (Wassinger et al., 2013).

Classification and mode of action

Despite the high degree of heterogeneity in the E-O domain across the family, in a phylogenetic study Rigali and colleagues observed a clear clustering of four E-O types, suggesting the existence of at least four E-O ancestor domains that became fused to the common HTH DNA-

binding domain. This clustering eventually led to the classification of GntR proteins into its several subfamilies (i.e. FadR, HutC, MocR, YtrA) which, since then, have expanded to a total of six subfamilies, with the inclusion of AraR and PlmA subfamilies (Rigali et al., 2002).

FadR is the largest of these subfamilies with a total of almost 50% FadR proteins representing the GntR regulators deposited in the Pfam database. Members of the FadR subfamily contain an all-helical C-terminal domain with an average of 160 amino acids. Many different FadR regulators have been linked to regulation of amino acid metabolism and diverse metabolic pathways (gluconate - GntR, aspartate - AnsR, pyruvate - PdhR, glycolate - GlcC, galactonate - DgoR, lactate - LldR or malonate - MatR) (Suvorova et al., 2015). The FadR subfamily further subdivides in two classes according to the number of α-helices at the C-terminal. The FadR sub-class has seven α-helices while the VanR sub-class only has six. It appears that the evolutionary origin of VanR is a direct modification of FadR as the loss of the α4-helix seems like the only feature separating them (Jain, 2015). FadR from *Escherichia coli* is one of the best characterized GntR regulators and one of the first GntR regulators to have their 3D structure resolved. FadR controls the expression of several genes in order to regulate fatty acid biosynthesis and degradation (DiRusso et al., 1999). The FadR regulator forms a dimer which binds to specific DNA sequences and also binds acyl-CoA at the E-O domain which causes dramatic conformational changes and impairs DNA binding (van Aalten et al., 2001).

The HutC subfamily is the second largest GntR subfamily, approximately 30% of GntR regulators, and is characterized by a C-terminal structured in α-helical and β-sheet arrangement. The HutC C-terminal domain also shares a similar fold as the enzyme chorismate lyase, suggesting that just like that enzyme the HutC E-O domain may bind small molecules like histidine, fatty acids, sugars and alkylphosphonates (Aravind and Anantharaman, 2003). Members of this subfamily have been implicated in regulation of N-acetylglucosamine utilization and conjugative plasmid transfer (König et al., 2009; Suvorova et al., 2015; Yang et al., 2006).

Members that constitute the MocR subfamily share two characteristic features. First, a notably large C-terminal with an average of 350 amino acids in length. Second, homology to the class I of aminotransferase proteins. Members of the MocR subfamily have similar need for a specific cofactor pyridoxal 5'-phosphate and assume a head-to-tail dimer conformation which are both traits of the class I aminotransferase group (Belitsky, 2004; Bramucci et al., 2011; Rigali et al., 2002; Sung et al., 1991).

Known members of the YtrA subfamily are not as numerous as the previous subfamilies and are characterized by a smaller C-terminal domain, containing only two α-helices and averaging only 50 amino acids. There is also a low amount of similarity between C-terminal of these

members suggesting either multiple ancestors or recombination events throughout evolution. This family appears to be linked to regulation of ATP-binding cassette (ABC) transport systems (Jain, 2015).

The PlmA subfamily is represented exclusively by cyanobacterial GntR transcription factors but no common functions have been linked to members of this subfamily so far. They show some phylogenetic proximity to YtrA and MocR suggesting a possible ancestry within these groups (Lee et al., 2003; Suvorova et al., 2015).

The last GntR subfamily is AraR. While members of AraR retain the conserved GntR N-terminal domain, it is fused with an unrelated C-terminal domain homologous to the distant GalR/LacI family. So far they have been implicated in regulation of proteins related to L-arabinose, rabinose-containing polysaccharides, xylose and galactose (Franco et al., 2006, 2007; Suvorova et al., 2015).

Considering the big size of this family of transcription factors, not many structural data is available for GntR members. However, at least one crystal structure of the FadR (FadR from *E. coli* - Pdb code 1H9T, 1HW1, 1HW2), HutC (YvoA from *B. subtilis* – Pdb code 2WV0), MocR (GabR from *B. subtilis* – Pdb code 4N0B), YtrA (CGL2947 from *Corynebacterium glutamicum* – Pdb code 2EK5) and AraR (AraR DNA-binding domain from *B. subtilis* – Pdb code 4EGY, 4EGZ, 4H0E) subfamilies were resolved, although only the structures of FadR and AraR were resolved in complex with DNA (Figure 10).

A recent comparative-predictive study that assessed the frequency of transcription factor amino acid-DNA contacts within members from FadR, HutC and YtrA, predicted Arg, Asn, Lys, Gln, Thr, Ser, Asp and Gly to account for the majority of the contacts, with Arg as the most frequent, which is coincident with already published data for other factors (Suvorova et al., 2015). GntR regulators typically bind DNA sequences in a dimeric unit, where each monomer binds half of the recognition site. The head-to-head and anti-parallel configurations are observed within GntR dimers (Suvorova et al., 2015).

While it may not be reasonable to expect a direct correlation of the amino acid composition of a transcription factor and the recognized DNA sequence, it is reasonable to expect some degree of similarity between recognized sequences of members from a specific family or subfamily. A study compiled several known DNA sequences bound by members across the GntR superfamily and determined the consensus palindromic sequence: 5'-$(N)_y$GT$(N)_x$AC$(N)_y$-3' (Rigali et al., 2002). Analyses with data from specific subfamilies allowed to define the FadR 5'-t.GTa.tAC.a-3' and HutC 5'-GT.ta.AC-3' consensus recognition sequences. This indicates that GntR have a preference for a relatively short symmetric sequence. Several identified members (e.g. AphS, BphS, FarR, FucR, NagQ, NanR) do not follow this trend and bind

unrelated sequences even without any sequence symmetry (Arai et al., 1999; Condemine et al., 2005; Hooper et al., 1999; Quail et al., 1994; Watanabe et al., 2000; Yang et al., 2006).

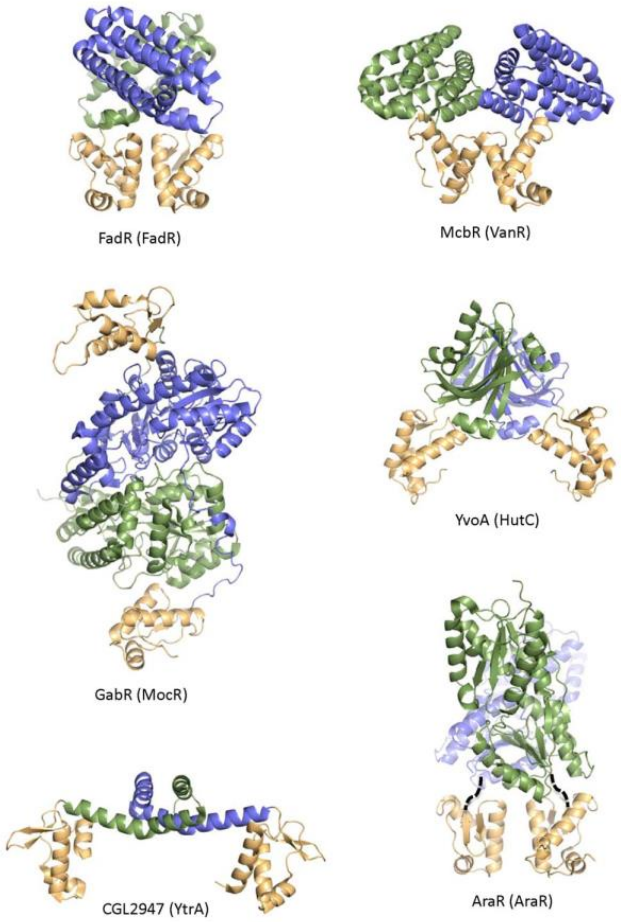

Figure 10 - Structures of representative member of different subfamilies of the GntR family showing the diversity in the effector binding domains. The name of subfamily is in parenthesis. The transcription factors are dimeric with the two DNA binding domains in each structure colored orange. The effector binding domains are in green and purple for the two monomers. (Adapted from Jain, 2015)

The Accessory Gene Regulator (Agr) system

Discovery and general features

The Accessory gene regulator (Agr) system is a bacterial quorum sensing module encoded by a four-gene operon (*agrBDCA*). The system has been well characterized in *S. aureus* where it represents one of the most important units of virulence regulation, but homologues have now been identified in other Gram-positive bacteria including *Lm* (Wuster and Babu, 2008). In this system the quorum sensing module is paired with a classical two-component system (Figure 11). Mechanistically, the Agr system expresses a pheromone peptide encoded by *agrD* which

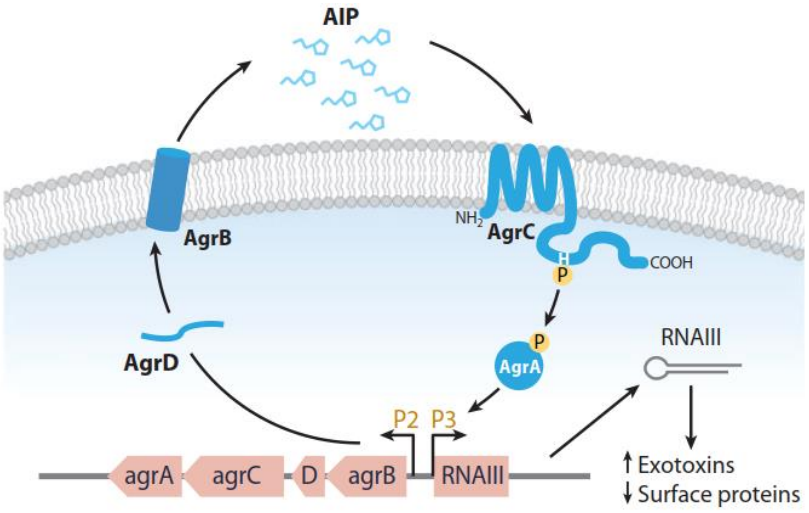

Figure 11 – Agr system of *S. aureus*. The AgrD peptide, processed by AgrB, is secreted in the form of a tailed thiolactone ring (AIP), which acts on the transmembrane receptor domain of AgrC, inducing phosphorylation of the cytoplasmic HPK domain. The phosphate is transferred to AgrA, which activates transcription from the two *agr* promoters. The P2 promoter drives the auto activation circuit, and the P3 promoter drives transcription of RNAIII, which is the regulatory effector of the system. (Adapted from Novick and Geisinger, 2008)

is processed into a mature auto-inducing peptide (AIP) and exported out of the cell by the transmembrane endopeptidase AgrB. Also at the membrane, the transmembrane histidine kinase AgrC (two-component sensor kinase) recognizes and binds the pheromone AIP and transmits the signal intracellularly by phosphorylation of the cytoplasmic AgrA (two-component response regulator) which carries out diverse regulatory functions (Thoendel et al., 2011). This system is thought to be very sensitive since it has been shown that a single bacterium enclosed in a phagosome-like compartment can experience Agr quorum sensing and reprograming of gene expression (Carnes et al., 2010). The *agr* locus encompasses the *agrBDCA* operon but can have additional transcripts. In fact, in *S. aureus* the locus is composed by a transcript encoding the operon genes (RNAII) and a second one (RNAIII) transcribed in the opposing direction, both under positive control of AgrA (Janzon et al., 1989; Novick et al., 1995). RNAIII is the major downstream effector of the system and regulates the expression of virulence factors and also other regulators, but AgrA also activates other promoters outside of the *agr* locus (Novick et al., 1993; Queck et al., 2008). Regulation by Agr includes a wide range of genes whose expression is controlled at the transcriptional level, mRNA stability or translation initiation. Regulation is mediated by direct promoter binding by AgrA, RNAIII binding to target mRNAs or through interplay between RNAIII and the transcription regulator Rot (Thoendel et al., 2011). Agr is well known for regulating virulence factors including secreted toxins, enterotoxins, proteases, lipases, superantigens, capsule components and urease and to downregulate the expression of surface virulence factors implicated in adhesion and antibody binding. However Agr is also linked to regulation of other cellular functions like metabolic

pathways and nutrient transport (Gray et al., 2013). Since AgrA binds and activates expression of both RNAII and RNAIII the system is under its own positive autoregulation. The regulon of AgrA is estimated to be around 100 protein-encoding and sRNA genes, most of which are actually repressed by AgrA. Because of this, it has been proposed that instead of a direct regulation there may also be additional interplays between AgrA and other regulators (Queck et al., 2008; Thoendel et al., 2011).

The Agr system is itself under constant regulation and the number of transcription factors with an *agr* phenotype is high. Aside from the system autoregulation Agr expression is also influenced by environmental factors and stresses like pH and carbon source (Regassa et al., 1992; Seidl et al., 2006), both positive (SarA, SarU, SarZ, MgrA) and negative (σ^B, CodY, Rsr, SarX) control by other regulators and also by host factors (Gray et al., 2013; Thoendel et al., 2011). Interestingly, while other positive regulators can have dramatic influence on Agr expression, the lack of the AgrA protein itself completely abolishes the expression of both RNAII and RNAIII (Reyes et al., 2011). As a quorum sensing system, Agr in *S. aureus* monitors population density and regulates gene expression accordingly. This is thought to be true both in the environment and the host. Biofilm formation is an important mechanism of environmental persistence and infection promotion that generally is dependent on quorum sensing, but this relationship is far from clear. When Agr is defective *S. aureus* forms a stronger more adhesive biofilm but Agr has a heterogeneous expression pattern on biofilms that is very hard to characterize (Novick and Geisinger, 2008; Yarwood et al., 2004). Whereas Agr has been shown to be important for infection and many reports have demonstrated attenuated phenotypes of *S. aureus agr* mutant strains in infection models, the implication of quorum sensing in infection is not fully understood (Abdelnour et al., 1993; Cheung et al., 1994; Gillaspy et al., 1995; Wright et al., 2005). It has been proposed that quorum sensing allows bacteria to stay under the radar of the immune surveillance by keeping the expression of immunogenic virulence determinants low until the population grows sufficiently large (Novick and Geisinger, 2008).

Agr system in *Lm*

Genome analysis of *Lm* EGD-e led to the identification of a complete *agr* operon with all four genes arranged as described in *S. aureus* (*agrBDCA*). The degree of protein identity between the two species is 28%-AgrB, 30%-AgrC, 41%-AgrA and no similarity except for size for AgrD, a degree of difference which is common for pheromone peptides even within species (Figure 12) (Autret et al., 2003; Ji et al., 1997; Zetzmann et al., 2016). Bioinformatic analyzes predict that AgrC has a similar structure and membrane topology as its *S. aureus* counterpart. The

locus is also present in other *Lm* strains and also non-pathogenic species like *L. innocua* suggesting a role in the environment (Autret et al., 2003). The major difference in the *Lm* Agr system is the complete absence of an RNAIII homologue. Not only there is no sequence homology found at the vicinity of the locus nor anywhere in the genome, also there is an ORF upstream of *agr* encoded in the same orientation as the operon, as opposed to the divergent nature of RNAIII (Autret et al., 2003).

So far the Agr system has been shown to play various roles in *Lm* stress response adaptation and pathogenicity. Because the *agr* locus lacks the RNAIII transcript the system downstream regulatory mechanism is thought to be mediated solely by the effector AgrA. However, and despite the fact that Agr seems to mediate a large transcriptional response involving hundreds of genes, no direct targets for AgrA have been identified (Riedel et al., 2009; Rieu et al., 2007). Interestingly, despite the fact that Agr is the only quorum sensing system known to be encoded by *Lm,* studies have demonstrated that unlike in other bacteria Agr expression in liquid culture and biofilms is very heterogeneous and that the quorum sensing does not appear relevant for monitoring *Lm* population density nor has a strong influence on population growth (Autret et al., 2003; Garmyn et al., 2011; Gray et al., 2013). In this sense the *Lm* Agr system may be another case of quorum sensing systems that display alternative functions not limited to density sensing and response (Platt and Fuqua, 2010). More specifically, there have been demonstrations of Agr involvement in *Lm* competition and survival in the environment, the formation of biofilm and virulence *in vitro* and *in vivo*.

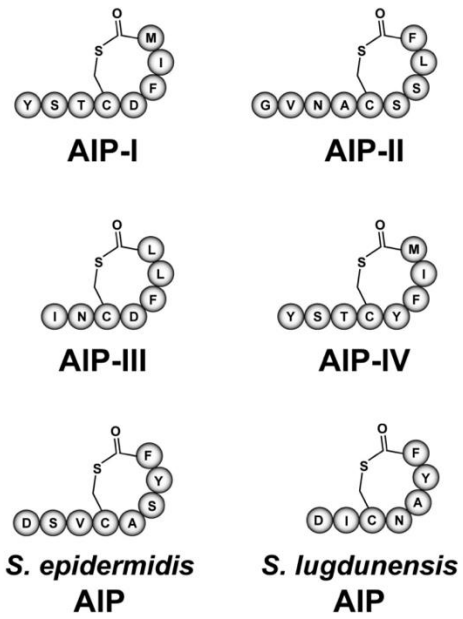

Figure 12 - Structures of staphylococcal autoinducing peptides (AIPs). Four *S. aureus* AIPs types and the most common *Staphylococcus epidermidis* AIP and the predicted structure of the *S. lugdunensis* AIP. (Adapted from Thoendel et al., 2011)

The role of Agr in *Lm* competition and survival has been elucidated in the soil environment. Vivant and colleagues recently demonstrated that mutants for AgrA and AgrD showed a similar growth to the parental strain when incubated in several different types of sterile soil, but when the indigenous microflora was not eliminated the mutant strains showed a clear disadvantage and their population declined over time. In addition to this, the sterility state of the soil had a significant impact on the transcriptional changes observed between the parental and Agr mutant strains (Vivant et al., 2014, 2015). This suggests an important role of Agr in the saprophytism of *Lm*.

The implication of Agr in *Lm* biofilms has been demonstrated multiple times. The formation of biofilms is an important trait of saprophyte and pathogenic bacteria and consists on the transition of a planktonic into a sessile state by the formation of a specialized structure strongly adhered to a surface or interface (Figure 13). After adhering to a surface bacteria produce and secrete polymers that form an extracellular matrix that grants both tight adherence of the formed microcolonies to the surface and a better protection against a variety of external factors and aggressions (e.g. ultraviolet rays, toxic metals, desiccation, salinity, antimicrobials, disinfectants, etc.) (Carpentier and Cerf, 2011; Da Silva and De Martinis, 2013). Whereas the role of *Lm* biofilm inside the host is still mostly unclear, in food processing environments biofilms constitute a form of persistence and dissemination of *Lm.* Persistent *Lm* has been isolated from machinery, conveyor belts, cutting boards, walls, drains and several other surfaces and tools and failure to eliminate an *Lm* biofilm can lead to constant contamination of food products and an increased risk of outbreak (Colagiorgi et al., 2017; Langsrud et al., 2004; Da Silva and De Martinis, 2013). Whereas in *S. aureus* the Agr system is known to negatively regulate biofilm formation several studies have demonstrated how *agr* mutants of *Lm* develop smaller biofilms instead (Riedel et al., 2009; Rieu et al., 2007, 2008). This suggests a positive correlation between *Lm* biofilm and Agr activity. The expression pattern of Agr within biofilm forming *Lm* populations is very heterogeneous, with higher expression levels from the surrounding elongated-chains rather than the densely populated microcolonies where AIP concentration would be expected to be higher (Rieu et al., 2008). While population density may not be the main connection between Agr and *Lm* biofilm formation, further research is needed to elucidate this question.

The link between Agr and *Lm* virulence has been demonstrated both *in vitro* and *in vivo*. Mutants for components of Agr have showed limited ability to adhere and invade eukaryotic host cells and also revealed an attenuated phenotype of infection in the mouse model (Autret et al., 2003; Riedel et al., 2009). These phenotypes could be partially associated to less expression of internalins InlA and InlB by the mutants but the details of the link between Agr

and *Lm* virulence are mostly unknown (Garmyn et al., 2012; Riedel et al., 2009). Whether the Agr-mediated quorum sensing has a role on *Lm* infection or not also requires elucidation.

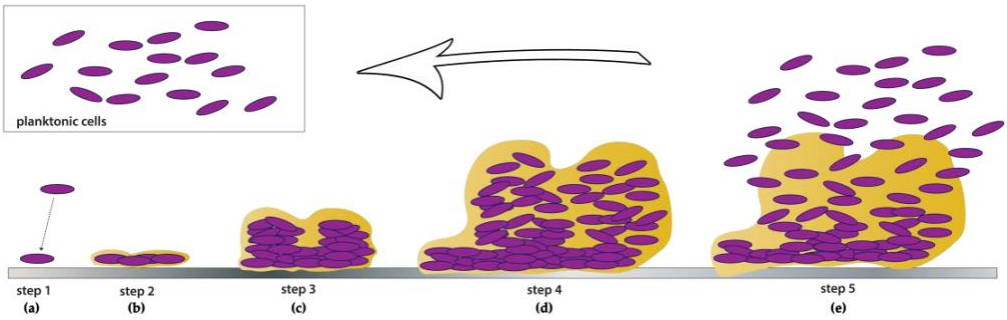

Figure 13 - Schematic representation of the biofilm development stages. (a) The first step involves planktonic cells reversible attachment to surfaces; **(b)** the adhered cells begin to form a monolayer and to produce extracellular matrix; **(c)** the cells within the self-produced extrapolymeric matrix continue to grow and form multilayered microcolonies; **(d)** cells are irreversibly attached to the surface and embedded in the matrix: the biofilm is mature; **(e)** in the last stage of biofilm formation, cells are able to detach from the biofilm and to return in planktonic form, ready to colonize new surfaces. (Adapted from Colagiorgi et al., 2017)

Unlike the extensive knowledge regarding how Agr is regulated in *S. aureus* little is known regarding *Lm*. There is evidence that points towards an autoregulation of the system by AgrA and also transcriptomic studies that show that environmental cues like temperature can modulate the expression of *agr* and its regulon (Riedel et al., 2009; Rieu et al., 2007). Nevertheless, no regulators of the *Lm* Agr system have been identified and characterized yet and the knowledge on the regulatory network upstream of Agr remains one of the biggest missing links.

Secretion systems and bacterial virulence

The secretion of proteins is an important feature of saprophytic and pathogenic bacteria. From quorum sensing (like the Agr system) to the release of potent toxins (like LLO), protein secretion gives bacteria the ability to sense, modify and adapt to their surroundings both in the environment and inside the host (Costa et al., 2015). Bacteria deploy several highly specialized and sophisticated molecular machinery known as secretion systems on their cell envelope, which process and export different types of factors, including virulence factors, without compromising the cell barrier defense (Keyzer et al., 2003). Major routes of protein translocation like the general secretion pathway (Sec pathway) and the twin arginine translocation (Tat pathway) are widely distributed and have a large range of target effectors. Alternative routes consist of much less conserved and more specialized secretory complexes

such as the fimbrilin protein exporter (FPE) system, the flagellar export apparatus (FEA), the ESX-1 system or prophage holins (Bottai et al., 2015; Carvalho et al., 2014; Desvaux and Hébraud, 2006). Whereas *B. subtilis* has recently become a model for protein translocation studies in Gram-positive bacteria, most of the information on the function and component characterization of the major systems was obtained from studies performed on *E. coli* (Keyzer et al., 2003; Schneewind and Missiakas, 2012; Wely et al., 2001). The Sec system is the major bacterial transporter system for unfolded proteins. It is formed by a membrane translocase complex composed by two main components, the peripheral adenosine triphosphatase (ATPase) SecA that functions as the cytosolic molecular motor of protein translocation through ATP hydrolysis and the transmembrane SecYEG protein-conducting channel. Newly synthesized peptides that contain a classical signal peptide are recognized by the chaperone SecB that directs it to SecA for translocation (Keyzer et al., 2003). With the widespread application of whole-genome sequencing the Sec system was readily identified throughout the Gram-positive bacteria group. Indeed, Gram-positive bacteria encode homologues for most of the components of the Sec pathway and are thought to display a secretion pathway similar to that described for *E. coli*. Some differences in Gram-positive bacteria are notable like expression of alternative accessory secretion genes *secA2, secY2* or *prsA* and the absence of a *secB* homologue (Schneewind and Missiakas, 2012; Sibbald et al., 2006). Specialized secretion systems appear to be much more diverse in Gram-negative bacteria where six different secretion systems (type I, II, III, IV, V and VI secretion systems) have been described (Costa et al., 2015). However, a type VII secretion system that was first described in mycobacteria has not been observed in Gram-negative but rather appears to be restricted to Gram-positive bacteria. This secretion system is referred to as the ESX-1 secretion system and homologues have been identified in several Gram-positive bacteria genus (Baptista et al., 2013; Burts et al., 2005; Costa et al., 2015; Garufi et al., 2008; Stanley et al., 2003).

The ESX-1 secretion system

The ESX-1 secretion system, also known as WXG100 or type VII secretion system, is a Sec-independent cell envelope secretory module of Gram-positive bacteria. Proteins secreted by this system contain an alternative signature motif to the classical signal peptide. This motif consists of a Trp-X-Gly (WXG motif) sequence which is linked to ≈100 amino acid-long helical proteins, hence the name WXG100. The system was first identified in *Mycobacterium tuberculosis* after the virulence factor ESAT-6, a member of the WXG100 family and a T cell antigen, had been detected as a highly secreted protein without harboring a typical signal peptide sequence (Sørensen et al., 1995). Confirmation of secretion of ESAT-6 and its

WXG100 partner CFP-10 by ESX-1 was followed by observations of absence or partial non-functional ESX-1/ESAT-6 systems in attenuated *Mycobacterium* strains, which started to establish a link between ESX-1 and virulence (Lewis et al., 2003; Simeone et al., 2015). Since then other paralogues have been identified inside the *Mycobacterium* group, ESX-2 – ESX-5, that share the common ESX-1 system basic structure: ESX conserved components (Ecc), ESX-secretion associated proteins (Esp), secreted Esx proteins (*esx* genes) and proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motif-containing proteins (Figure 14) (Simeone et al., 2015). The main components of the secretion machinery are the cytosolic (EccA) and membrane (EccC) ATP-binding proteins and other accessory transmembrane proteins (EccB, EccD, EccE) that mediate protein translocation (Bitter et al., 2009; Brodin et al., 2006).

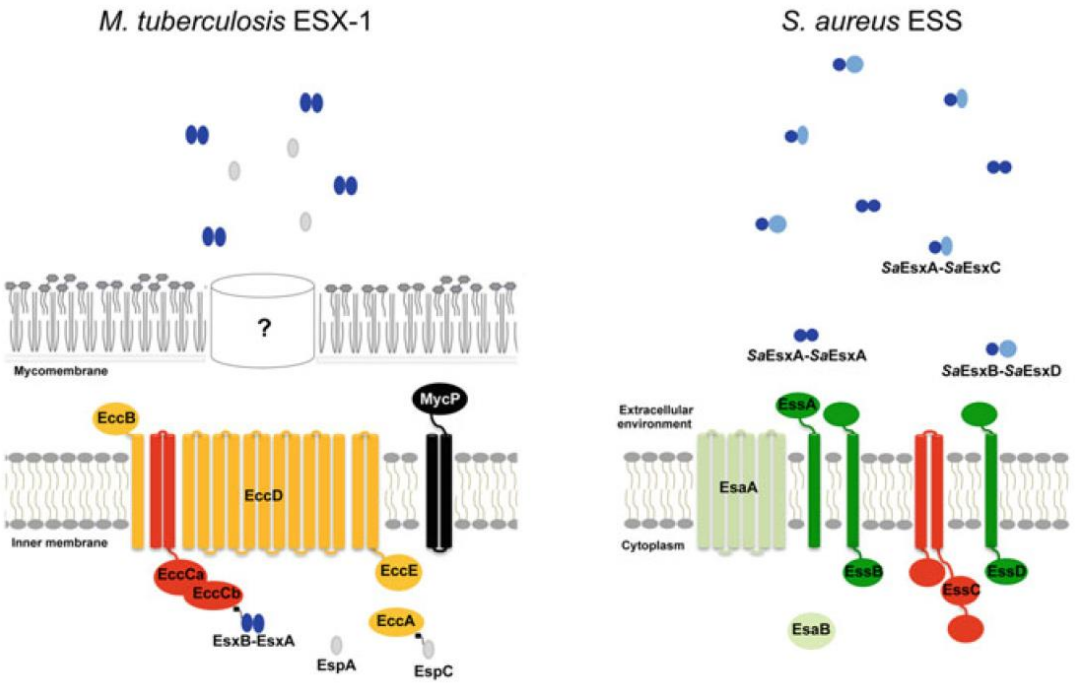

Figure 14 - Working model of the type VII- and type VII-like secretion machineries of *M. tuberculosis* (ESX-1) and *S. aureus* (ESS). (Adapted from Bottai et al., 2015)

The roles of ESX-1 secretion system in virulence were initially demonstrated in *M. tuberculosis* where strains with a fully functional ESX-1 system were able to escape the vacuole of macrophages and dendritic cells while strains with truncated or absent ESX-1 systems remained trapped inside the vacuole (Bottai et al., 2015; Simeone et al., 2015). Further research on the ESX-1 system led to the identification of functional homologue systems in *Bacillus, Clostrdium, Listeria, Staphylococcus, Streptococcus* and *Streptomyces*. Loci

encoding these systems share at least one *esx* gene and a gene encoding a typical ESX ATPase family member but many variable genes occur between genus, which may confer distinct functionalities and roles for each genus or species (Bottai et al., 2015). For example, the ESX-1 homologue system of *S. aureus* (Figure 14) is composed by genes that encode two canonical ESX-1 substrates (EsxA, EsxB), a FtsK/SpoIIIE-like ATPase (EssC) anchored at the membrane and essential for the secretion machinery, genes coding for membrane-embedded proteins (EssA, EssB, EssD) required for secretion of ESX-1 substrates, two additional staphylococci-specific ESX-1 substrates (EsxC, EsxD) and two ESX-1 activity modulators (EsaA, EsaB) (Anderson et al., 2011, 2013; Burts et al., 2005; Chen et al., 2012). The EsxA and EsxB substrates of *S. aureus* share some similarities with their *M. tuberculosis* counterparts (ESAT-6 and CFP-10) like the WXG motif and a co-dependent translocation. However, they do not appear to interact with each other, rather EsxA monomers form a dimer or interact with EsxC and EsxB interacts with EsxD (Anderson et al., 2013). Roles of the *S. aureus* ESX-1 system in pathogenesis have been described by characterization of mutants for the secretory machinery or secreted effectors which showed significant attenuation of virulence (e.g. reduction in the ability to establish kidney or liver abscesses (Anderson et al., 2011; Burts et al., 2005, 2008), affected nasal colonization and virulence in a murine lung pneumonia model (Kneuper et al., 2014), interference with host cell apoptotic pathways and bacterial release from host cells and survival (Korea et al., 2014), role in establishing a persistent infection (Burts et al., 2008)). Notably, the only report on ESX-1 and *Lm* pathogenicity indicates the system has no role on *Lm* virulence (Way and Wilson, 2005).

CHAPTER II – PROJECT PRESENTATION

Project goals

Our research group dedicates to the investigation of the molecular and cellular facets of host-pathogen interactions. To achieve this we use *Lm,* one of the best well-studied foodborne pathogens and an outstanding model for advancement in the fields of microbiology, infection and cell biology (Cossart, 2011; Cossart and Lebreton, 2014). One of our main goals is to characterize the *Lm* virulence mechanisms deployed to invade, survive and multiply within the host.

The central aim of this PhD thesis was to identify and characterize new mechanisms of virulence regulation used by *Lm* to promote infection.

Lm success as a pathogen is due in particular to its ability to cross major physiological barriers (i.e. intestinal, blood-brain and the fetoplacental barriers) (Lecuit, 2007), to invade phagocytic and non-phagocytic cells and to resist intracellular killing by macrophages (Sousa et al., 2005). The adaptability of *Lm* to the host environment is fundamental for the infection process and requires mechanisms for the tight and coordinate regulation of virulence factor expression. In a previous study we provided the first comprehensive view of the genome expression of this pathogen in deeper organs of infected mice (Camejo et al., 2009). In that study we showed that about 20% of the *Lm* genome is differentially expressed, while colonizing the mouse spleen. We also demonstrated that the differential expression of the *Lm* genome *in vivo* is coordinated by a complex regulatory network. In particular, *in vivo Lm* upregulates the expression of the two major virulence regulators, PrfA and VirR, as well as their downstream effectors (Camejo et al., 2009). Interestingly, we found that *Lm* differentially expresses other putative regulator-encoding genes during mouse infection, which could be relevant for virulence (Camejo et al., 2009). In the first part of this thesis we present the characterization of two *Lm* putative virulence transcription regulators (MouR and Lmo0443) that were identified through our *in vivo* transcriptomic data coupled with *in silico* analyses (see section below). Here, we constructed several *Lm* mutant strains to study the roles of the putative regulators in virulence regulation *in vitro* and *in vivo* and designed a genome-wide transcriptomic approach to identify and characterize the genes controlled by each regulator. On the second part of this thesis we present our study of *Lm* virulence regulation by protein secretion by the ESX-1 secretion system. We constructed several *Lm* mutant strains in order to characterize the roles of the secretory components and secreted factors of ESX-1 in *Lm* virulence.

Selection of *Lm* putative virulence transcription regulators

As already mentioned, a part of this project focused on the characterization of two *Lm* virulence regulators. Here we briefly describe how we selected MouR and Lmo0443 for study.

The search and selection of novel putative virulence regulators was mostly based on thorough analysis of our *in vivo* transcriptomic data (Camejo et al., 2009), analysis of other genome-wide transcriptomic studies in the literature and *in silico* analyses for protein function prediction. In the table below is represented a summary of the preliminary list of some putative transcription regulators of *Lm* that were initially considered for study.

The major criteria taken into account for gene selection were the following:

- Differential expression levels as shown by *in vivo* and *in vitro* transcriptomic studies (see table below for details on transcriptomic data).
- Genes whose products encode putative transcription factors (by *in silico* analysis).
- Genes whose products and functions have not been extensively characterized.
- Genes whose predicted role could potentially be linked to *Lm* virulence (e.g. encoded protein has high similarity to well characterized bacterial virulence regulator).
- Genes that do not have an identified ortholog in the non-pathogenic *L. innocua*.

Summary of pre-selection of *Lm* putative virulence encoding-genes

Gene	*L. innocua* ortholog	Spleen[(a)]	Blood[(b)]	Intestine[(b)]	Macrophage[(c)]	Caco-2[(c)]
lmo0443	✓	+	+	-		
lmo0459				-		+
lmo0597	✓	+	+			
lmo0612	✓	+				
lmo0651	✓	+	+	+		
lmo2460	✓		-	-	-	-
lmo2672		-			+	

Differential gene over (+) or under (-) expression in *Lm* recovered from the following conditions as compared to *Lm* grown in BHI: (a) Spleens of *in vivo* infected mice (Camejo et al., 2009); (b) *ex vivo* human blood inoculation and intestine lumen of *in vivo* infected mice (Toledo-Arana et al., 2009); (c) *in vitro* infection of mouse macrophage (Chatterjee et al., 2006) and human Caco-2 cell lines (Joseph et al., 2006).

We then constructed deletion mutants for each of the pre-selected genes and tested their capacity to invade eukaryotic host cells and infect mice, as compared to the *Lm* wild type strain. For the purpose of this thesis, the two putative regulators MouR (previously Lmo0651) and

Lmo0443 were ultimately selected for further investigation based on the observed attenuated *in vitro* and *in vivo* phenotypes, which will be discussed in detail.

CHAPTER III – RESULTS

Note: In this chapter the results produced in this work are organized in three different parts. In each part the results are presented together with their respective discussion.

PART I and **PART III** are written and presented in the article format and consist of unpublished and published data, respectively.

PART II is not in the article format and consists of unpublished data from ongoing work.

All the methodology and resulting data from **Part I** and **Part II** were executed and obtained by the author, with the exception of the crystallization and structure determination of MouR and the construction of the *Lm ΔagrC* strain which were performed in a collaboration with Dr. Nuno Santos, Dr. Johnny Lisboa and Dr. João Cabral and Dr. Hannu Korkeala and Dr. Anna Pöntinen, respectively. The methodologies of Western blot analysis and intracellular multiplication from **Part III** were executed by the remaining main authors of the research article.

PART I

MouR controls the expression of the *Listeria monocytogenes* Agr system and mediates virulence

MouR controls the expression of the *Listeria monocytogenes* Agr system and mediates virulence

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Abstract

The foodborne pathogen *Listeria monocytogenes* (*Lm*) can cause invasive infection in susceptible animals and humans. To survive and proliferate within hosts, this facultative intracellular pathogen needs to tightly coordinate the expression of a complex regulatory network, including virulence factors. Here, we identify and characterize MouR, a novel virulence regulator of *Lm*. Through RNAseq transcriptomic analysis, we characterized the MouR regulatory network and demonstrated how MouR positively controls the expression of the Agr system (*agrBDCA*) of *Lm*. Resolving MouR 3D structure revealed a dimeric DNA-binding transcription factor belonging to the VanR class of the GntR superfamily of regulatory proteins. We showed that by direct binding to the *agr* promoter region, MouR ultimately modulates chitinase activity and biofilm formation. Importantly, we demonstrated by *in vitro* cell invasion assays and *in vivo* mice infections the crucial role of MouR for *Lm* full virulence.

Introduction

Pathogenic bacteria depend on an arsenal of genetic elements called virulence factors to successfully infect their host. From cell invasion to nutrient acquisition and subversion of the immune system, by exerting specific functions each virulence factor plays crucial roles in the infectious process (Wu et al., 2008). While the expression of such factors is fundamental at a given time, constant or overexpression of the virulence gene pool can become energetically wasteful or even harmful for the pathogen (McKenney and Kendall, 2016; Vasanthakrishnan et al., 2015). Thus, the tight regulation of virulence factors becomes a crucial mechanism for pathogen survival and fitness. Transcriptional regulators of virulence respond to various signals, either environmental or from the host, and accordingly trigger a switch in the virulence factor expression pattern (Kepseu et al., 2012; Papenfort and Vogel, 2010).

Listeria monocytogenes (*Lm*) is a Gram-positive bacterium and the causative agent of the systemic disease listeriosis. Although ubiquitous and commonly found growing in diverse environments, *Lm* switches from a saprophyte into a deadly pathogen (Camejo et al., 2009; Cossart and Toledo-Arana, 2008), currently holding the highest mortality rate among foodborne pathogens (EFSA Report, 2016). *Lm* is well equipped to survive the hostile conditions of the human digestive tract, invade both phagocytic and non-phagocytic cells, cross major biological barriers (intestinal, blood-brain and fetal-placental barriers), cause septicemia in immunocompromised individuals, infants and the elderly, and in the case of pregnant women severely infect the fetus (Camejo et al., 2011; Cossart, 2011; Swaminathan and Gerner-Smidt, 2007).

Virulence regulation of *Lm* greatly depends on the transcription regulator PrfA. By controlling the expression of a broad list of genes, including *Lm* major virulence factors, PrfA is regarded as the master virulence regulator (Freitag et al., 2009). Despite this, recent researches have unveiled several other important regulators such as σ^B (Oliver et al., 2010), VirR (Mandin et al., 2005), Hfq (Christiansen et al., 2004), MogR (Shen and Higgins, 2006), DegU and GmaR (Kamp and Higgins, 2009; Williams et al., 2005) which, to a lesser extent, contribute to the *Lm* virulence regulatory network.

The GntR family of proteins is a large group of transcription factors that is found in many diverse bacteria and associated with the regulation of diverse biological processes (Haydon and Guest, 1991; Suvorova et al., 2015). Members of this group share a basic structure of a DNA binding domain at the N-terminal and an effector binding/oligomerization (E-O) domain at the C-terminal (Hoskisson and Rigali, 2009). The N-terminal is highly conserved among members, with a characteristic helix-turn-helix (HTH) DNA-binding domain. In turn, the E-O domain shows higher diversity which is used to categorize the diverse GntR sub-families

(FadR, HutC, MocR, YtrA, AraR and PlmA) (Suvorova et al., 2015). FadR from *Escherichia coli* is one of the best characterized GntR regulators. It regulates fatty acid biosynthesis and degradation through activation and repression of several genes (DiRusso et al., 1999). FadR dimers bind to specific DNA sequences through the HTH domain and bind acyl-CoA at the E-O domain which causes dramatic conformational changes impairing DNA binding (van Aalten et al., 2001).

The accessory gene regulator (*agr*) locus encodes a bacterial communication system consisting of a quorum sensing module paired with a classical two-component system (Novick and Geisinger, 2008). The Agr system was first described in *Staphylococcus aureus* but has since been found in many other Gram-positive bacteria (Wuster and Babu, 2008). In *Lm*, it has been associated with survival and competitive advantage in soil, adhesion to surfaces and biofilm formation, invasion of mammalian cells, infectivity in the mouse model and global changes in gene expression (Autret et al., 2003; Riedel et al., 2009; Rieu et al., 2007; Vivant et al., 2014, 2015). Interestingly, despite being the only quorum sensing system described in *Lm,* it appears that monitoring population density is not its main function (Garmyn et al., 2011) and the role of its quorum sensing properties is mostly unknown (Gray et al., 2013). Also, no regulator of the Agr system has been described so far for *Lm*, its autoregulation being the only regulatory mechanism proposed (Riedel et al., 2009; Rieu et al., 2007).

We previously provided the first comprehensive view of the genome expression of *Lm* directly in deep organs of infected mice (spleen) (Camejo et al., 2009). This enabled us to identify and characterize novel virulence factors, otherwise difficult to predict (Carvalho et al., 2015; Pombinho et al., 2017; Reis et al., 2010). This study also showed that the *in vivo* differential expression of the *Lm* genome is coordinated by a complex regulatory network, in particular through the up regulation of the two major virulence regulators, PrfA and VirR, and their downstream effectors (Camejo et al., 2009). Interestingly, during mouse infection, *Lm* appears also to overexpress several new potential virulence regulators. Here, we identified a novel transcription factor – MouR – upregulated during infection and involved in the orchestration of *Lm* virulence regulation. Notably, we demonstrated that *mouR* encodes the transcriptional activator of the Agr system and is necessary for full virulence. We characterized MouR at the structural level, identified its regulon and demonstrated how it controls biofilm formation and *Lm* chitinase dependent immune evasion to promote infection.

Material and methods

Bacterial strains and growth conditions

Strains used are detailed in Table 1. *Lm* EGD-e (ATCC-BAA-679) and *E. coli* were routinely cultured in Brain Heart Infusion (BHI) and Lysogeny Broth (LB) (Difco), respectively. Cultures were grown at 37°C aerobically with shaking. BHI-agar and LB-agar (Difco) plates were used for growth on solid media. To draw growth curves of *Lm* strains, overnight cultures were diluted 1:100 in fresh BHI and absorbance of the culture ($OD_{600\ nm}$) was measured every 30 min. Antibiotics were added to the media whenever appropriated: ampicillin 100 µg/ml, erythromycin 5 µg/ml and kanamycin 50 µg/ml.

Cloning

Mutant construction and strain complementation. The deletion of *mouR* (*lmo0651*) from the EGD-e wild type strain was achieved by a double homologous recombination process with the suicide plasmid pMAD (Arnaud et al., 2004). The detailed procedure was previously described (Carvalho et al., 2015) and was performed with the primers listed in Table S1. Complementation of the Δ*mouR* mutant strain was performed by genomic reintroduction of the gene *in trans*, as described before (Camejo et al., 2009). Complementation was mediated by the *Lm* specific integrative plasmid pIMK (Monk et al., 2008), through the construction of pIMK(*mouR*) (Table 1), with the primers described in Table S1.

Plasmid for protein overexpression. The coding region of *mouR* was C-terminaly fused with a 6-His tag by in frame restriction enzyme cloning into the pET28a expression vector, using the primers detailed in Table S1, creating pET28a(*mouR*-6His).

Site-directed mutagenesis. To induce the substitution of MouR arginines Arg44 and Arg48 with alanine residues, site-directed mutations were performed on pIMK(*mouR*) and pET28a(*mouR*-6His) with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacture's recommendations and with the primers from Table S1, originating pIMK(*mouR*-Mut) and pET28a(*mouR*-Mut).

Bioinformatic analyses

Gene sequences were obtained from the Genbank database (Benson et al., 2013) and homologue searches were performed with the BLAST (Boratyn et al., 2013) tool. Search of conserved protein domains and prediction of protein function were performed with the web-based PROSITE (Sigrist et al., 2013) and NCBI's Conserved Domain Database (Marchler-

Bauer et al., 2017) tools. Comparative analyses of protein sequences was conducted in MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 (Kumar et al., 2016).

RNA isolation and RT-qPCR

Lm cultures were grown in BHI to an exponential phase ($OD_{600\ nm}$= 1.0) and total RNA isolation was done by the phenol-chloroform method described elsewhere (Milohanic et al., 2003), with modifications (Pinheiro et al., 2017). After bacteria lysis, isolation was performed with the TripleXtractor reagent (Grisp) following the manufacturer's recommendations. RNA samples were depleted for DNA by DNase treatment (Turbo DNA-free, Ambion) and RNA purity and integrity was verified by 1% (w/v) agarose gel electrophoresis and Experion Automated Electrophoresis System (Bio-Rad Laboratories) virtual gel analysis. RNAs were reverse-transcribed into cDNA with a random hexamer cocktail-based kit (iScript Kit, Bio-Rad Laboratories) and RT-qPCR was performed using the primers in Table S1 with the iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) and a real-time PCR detection system (iQ5, Bio-Rad Laboratories) with the following cycling protocol: 1 cycle at 95°C (3 min); 40 cycles at 95°C (10 s), 56°C (20 s) and 72°C (20 s). The recorded data was normalized to that of a reference housekeeping gene (16S rRNA) and analyzed by the comparative threshold (ΔΔCt) method.

RNAseq transcriptome

DNA-free total RNAs were depleted for predominant rRNA species by processing with MICROBExpress Bacterial mRNA Enrichment Kit (Ambion) according to the manufacturer's recommendations. Efficient enrichment was verified by Experion Automated Electrophoresis System (Bio-Rad Laboratories) virtual gel analysis and Qubit 3.0 Fluorometer (Thermo Fisher Scientific) analysis. Sequencing of mRNAs was performed using the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) and quality control for RNA fragmentation and library construction was assessed by Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and 2200 TapeStation (Agilent) analyses. Template preparation was achieved with an Ion Chef system (Thermo Fisher Scientific) and sample sequencing was done in triplicates with an Ion Proton System (Thermo Fisher Scientific). Generation of sequence reads and read trimming and filtering was done with Torrent Suite v4.4 software with a FileExporter v4.4 plugin for generation of FASTQ/BAM files. To assess differentially expressed genes between *Lm* WT and Δ*mouR* mutant strain, sequencing reads were aligned to the reference genome sequence of *Listeria monocytogenes* EGD-e (RefSeq: GCF_000196035.1, GenBank: GCA_000196035.1,

assembly ASM19603v1 -http://www.ncbi.nlm.nih.gov/assembly/GCF_000196035.1) using the aligner TopHat2 (Kim et al., 2013). After transcript assembly, the relative abundance of each transcript was estimated by calculation of the metric fragments per kilobase of transcript per million mapped reads (FPKM) (Trapnell et al., 2011) using Cufflinks and Cuffdiff tools (Trapnell et al., 2013). Statistical significance was attributed to transcripts with fold change of expression higher than 2 or lower than 0.5, a p-value below 0.5 and an FDR-adjusted p-value below 0.1. Genomic alignment and differential expression analysis was performed as a service by the company Bioinf2Bio (www.frombioinformatics2biology.com). Sequencing results are available at the GEO database (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE106833.

Protein purification

The purification of 6xHis-tagged MouR and MouR(R44/48A) (Table 1) proteins was performed by *E. coli* heterologous overexpression and chromatography as described elsewhere (Glomski et al., 2002) with some modifications. Briefly, the growth of *E. coli* BL21(DE3), harboring either pET28a(*mouR*-6His) or pET28a(*mouR*-Mut), for 3 h at 37°C with agitation in LB supplemented with 0.5 M IPTG were empirically determined as favorable conditions for high protein expression. One liter of culture was grown and lysed with a French Pressure Cell Press (Thermo) at 12000 psi. The proteins were trapped in a nickel-based matrix (Ni-NTA Agarose, Qiagen) and purified by chromatography through imidazole promoted protein elution in a low pressure liquid chromatography system (BioLogic DuoFlow QuadTec10 System, Bio-Rad). Lastly, imidazole was diluted out of protein suspension by membrane dialysis (Spectra/Por Dialysis Membrane, SpectrumLabs) and proteins were concentrated in solution by Vivaspin (Sartorius Stedim Biotech) column centrifugation. For the expression and purification of SeMet-MouR, *E. coli* BL21(DE3) transformed with pET28a(*mouR*-6His) were grown in 40 ml of LB overnight at 37°C, collected and washed three times with sterile deionized water and finally used to inoculate 1 l of SelenoMethionine Medium (Molecular Dimensions). The procedure and conditions were exactly the same as described for the native protein.

Crystallization and structure determination of MouR

Both native and SeMet-substituted MouR proteins were stored in 50 mM Tris-HCl pH 8.0, 300 mM NaCl. Native crystals were grown at 20°C from a 0.1:0.1 µl mixture of a 10 mg/ml protein solution with a crystallization solution composed of 1.5 M lithium sulfate, 100 mM sodium Hepes pH 7.5. SeMet-MouR crystals were grown from a mixture of 6 mg/ml protein solution

with a crystallization solution composed of 3 M NaCl, 100 mM Tris pH 8.5. Crystals were flash-frozen directly in liquid nitrogen. Native and SeMet diffraction data were recorded on beamlines Proxima 2A (Soleil, Gif-sur-Yvette, France) and ID29 (ESRF, Grenoble, France). The structure was determined by the SAD method (Single-wavelength anomalous dispersion) using the anomalous signal from the selenium element. Data were processed with XDS (Kabsch, 1993) in space group P41, with two copies per asymmetric unit. All expected Se sites (eight by monomer) were found with SHELXD using reflections in the 50–3.5 Å resolution range (Schneider and Sheldrick, 2002). Refinement of Se atom positions, phasing and density modification were performed with Phenix-EP. The high quality of the experimental phases allowed automatic building (Autobuild program) of most of the protein model. The model was refined against the 2.2 Å native data set using PHENIX (Adams et al., 2002). The final model contains residues 3-217 from both monomers. In addition, 85 water molecules, 10 sulfate ions, one 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 6 ethylene glycol could be modeled into the electron density maps. Statistics for data collection and refinement are summarized in Table 2.

Electrophoretic mobility shift assays (EMSAs)

Protein-DNA binding was setup in 20 µl reactions containing 240 ng of DNA synthesized with the primers described in Table S1, binding buffer (50 mM Hepes pH 7.5, 500 mM NaCl, 1mM EDTA, 0.1 mM DTT, 5% Glycerol, 0.01 mg/ml BSA) and growing amounts of purified protein as previously described (Pombinho et al., 2017). The DNA was first incubated in binding buffer for a few minutes followed by gentle mixing of the protein and incubation at room temperature for 20 min. The entire reaction volume was then loaded into a 10% acrylamide native gel and run in TAE buffer. The gel was stained for 10 min in a 0.01% GreenSafe Premium (NZYTech) TAE buffer solution and imaged in a GelDoc XR+ System (Bio-Rad Laboratories).

Chitin hydrolysis assay

Preparation of chitin and evaluation of chitinase activity was performed as described (Paspaliari et al., 2014). 5 g of shrimp shell chitin (Sigma-Aldrich) were pre-treated with 50 ml of HCl (37%) overnight. Treated chitin was adjusted to pH 8 with NaOH and pelleted at 8300 g for 5 min, washed with ultrapure water seven times and used to supplement LB-agar plates (6 mg/ml). 10 µl of *Lm* overnight cultures were spotted on the plates and incubated at 30°C for 6 days. Chitin hydrolysis was evaluated by measuring the radius of the degradation halo.

Biofilm formation

The ability of *Lm* to form biofilm was evaluated by the crystal violet turbidimetry assay (Christensen et al., 1985). Overnight cultures of *Lm* were diluted 1:100 in BHI and 100 µl (octaplicates) were transferred to sterile 96-well PVC plates (Corning) and incubated at 30°C for 20 h. Wells filled with BHI served as control. After the incubation period, the media was removed and loose bacteria were washed with distilled water three times. After air drying for 30 min the wells were filled with 150 µl of 0.5% crystal violet (BDH) for 45 min. Excess stain was washed three times with distilled water, the plate was air dried and biofilm-associated crystal violet was resuspended in 200 µl of 99% ethanol. 100 µl of each well was transferred to a new plate and the $OD_{595\ nm}$ was measured in a plate reader (µQuant, Biotek).

Cell invasion assays

Assays of invasion of human cell lines were performed as described (Reis et al., 2010). Briefly, cells were grown to confluent monolayers in Eagle's medium with L-glutamine (Lonza), supplemented with nonessential amino acids (Lonza), sodium pyruvate (Lonza) and 20% fetal bovine serum (FBS, Biowest) (Caco-2, ATCC HTB-37) or in DMEM with glucose (4.5 g/l) and L-glutamine (Lonza), supplemented with 10% FBS (HeLa, ATCC CCL-2 and Jeg-3, ATCC HTB-36). *Lm* was grown to an exponential phase, washed and inoculated at a multiplicity of infection of 75 for 1 h. Cells were incubated with medium supplemented with 20 µg/ml gentamycin (Lonza) for 1.30 h to eliminate extracellular bacteria, washed and finally lyzed with 0.2% Triton X100. Bacterial suspensions were serially diluted and plated on BHI-agar plates for CFU determination.

Animal infections

Animal infections were performed with 6 to 9 week-old specific pathogen-free female BALB/c mice (Charles River Laboratories) maintained at the IBMC animal facilities, in high efficiency particulate air (HEPA) filter-bearing cages under 12 h light cycles and in an *ad libitum* regiment of sterile chow and autoclaved water. Intravenous infections were performed by inoculation of 10^4 CFUs through tail vein injection as described (Cabanes et al., 2008). For oral infections mice were starved for 8-12 h before the procedure and inoculated with 2 x 10^9 CFUs (in PBS with 150 mg/ml $CaCO_3$) by gavage under light anesthesia. Mice were sacrificed by general anesthesia 72 h post-infection and the liver and spleen of each animal were aseptically removed and homogenized in PBS. Organ homogenates were serial diluted and plated in BHI-agar plates for CFU counting. 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 Alimentação e Veterinária, the Portuguese authority for animal protection, under license 015302.

Statistical analyses

Statistical analyses were performed with the software Prism 7 (GraphPad Software). Means of two groups were compared by unpaired two-tailed Student's t-test. Differences with a calculated p-value above 0.05 were considered non-significant and statistically significant differences were noted as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

lmo0651 encodes a putative DNA-binding transcription regulator

In a previous study, we reported the *in vivo* transcriptome profiling of *Lm* while infecting the mouse spleen and identified the gene *lmo0651*, predicted to encode a transcriptional factor, as highly expressed during spleen infection when compared to growth in BHI medium (Camejo et al., 2009). Through bioinformatics and database analysis, Lmo0651 was predicted as a transcriptional regulator protein containing a putative typical GntR winged HTH DNA-binding domain (Suvorova et al., 2015) (Figure 1A). Protein sequence alignments showed that although Lmo0651 appears to be fairly conserved across the *Listeria* genus, found in the genome of several pathogenic and non-pathogenic species and serogroups (Figure 1B), it has no close relatives outside the genus, the closest hit being an annotated "DNA-binding transcriptional regulator, GntR family" from *Isobaculum melis*.

MouR is the transcriptional activator of the Agr system

To reveal the regulator role of Lmo0651 and identify genes under its control, we designed an RNAseq-based experiment to assess the genome-wide gene expression from both *Lm* wild type EGD-e (WT) and the Δ*lmo0651* deletion mutant in exponential growth phase in BHI at 37°C. When compared to the WT strain, six transcripts (*agrD, agrB, agrC, agrA, fruA, lmo0278*) appeared less abundant in the Δ*lmo0651* mutant, while only one (*lhrA*) was more abundant (Figure 2A), suggesting that Lmo0651 acts mainly as a transcriptional activator. Four of the corresponding genes (*agrD, agrB, agrC* and *agrA*) are clustered in an operon that encodes the components of the Agr quorum sensing system of *Lm* (Garmyn et al., 2009; Gray et al., 2013), that plays a role in survival in the environment, biofilm formation and in virulence (Autret et al., 2003; Riedel et al., 2009; Rieu et al., 2007; Vivant et al., 2015). *lrhA* is a non-coding sRNA (sRNA) negatively regulated by Agr (Paspaliari et al., 2014) and known to regulate several genes including the chitinase coding gene *chiA* through mRNA binding and interference with ribosome recruitment (Nielsen et al., 2009, 2011). In addition to genes related to the Agr system, *fruA* and *lmo0278* are two genes also less expressed in absence of Lmo0651 (Figure 2A) and are related to sugar uptake, where the former encodes one of the components of a fructose-specific phosphotransferase system and the latter encodes a maltose uptake ABC transporter (Deutscher et al., 2014; Gopal et al., 2010). These genes seem to be essentially related to metabolic pathways and, furthermore, they appear to have a complex dynamic type of regulation since they seem prone to be found regulated in comparative genome-wide transcriptomes (Camejo et al., 2009; Chatterjee et al., 2006; Joseph et al., 2006; Toledo-Arana et al., 2009).

We further confirmed our results by RT-qPCR. We selected a subset of up- and down-regulated genes and performed qPCR on cDNA from WT and Δ*lmo0651* bacteria grown to exponential phase. RT-qPCR results and RNAseq data exhibited a very strong correlation coefficient (R^2=0.93) (Figure 2B), validating the differential expression levels detected by transcriptomics.

Together, these results pointed Lmo0651, renamed MouR, as the transcriptional activator of the Agr system.

Overall structure of MouR

To further explore the function of MouR we expressed and purified MouR and resolved its 3D-structure (Figure 3). The crystal structure of MouR was determined by single-wavelength anomalous dispersion (SAD) using SeMet-substituted protein. The atomic model was refined against a native data set to 2.2 Å resolution (Table 2). The final model includes residues Asn3 to Arg217 for chains A and B and three histidines from the His-tag for chain B. The protein is a member of the GntR superfamily of dimeric transcription factors and is composed by two domains (Figure 3A). The N-terminal domain of MouR (Asn3-Cys73) is a winged-helix dsDNA-binding domain, characteristic of the GntR family and includes the canonical HTH DNA-binding motif followed by a β-hairpin (α1-α2-α3-β1-β2). The FadR C-terminal putative regulatory domain (FCD domain), encompassing residues Val75-Arg217, contains an antiparallel array of six α-helices (α4-α5-α6-α7-α8-α9) that form a barrel-like structure. The presence of only six α-helices within the FCD classifies MouR as a member of the VanR subclass of the FadR family of GntR regulators. MouR forms a homodimer in the crystal (superposition of each monomer yields a RMSD of 1.5 Å, http://zhanglab.ccmb.med.umich.edu/TM-align/) where the interface is mediated exclusively by helix α4 of the FCD (Figure 3B and 3C) (Zhang and Skolnick, 2005) burying a surface of ~ 1150 $Å^2$ per monomer (calculated with PISA server service; http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html). Residues that contribute to the dimerization are listed in Table S2, together with an estimate of the accessibility of individual residues in the dimer versus the monomer. Interface residues are located principally on α4 and the loop between α6 and α7 (Figure 3C). The interface involves a hydrophobic core (Ile89, Phe90, Ala93 and Ile152) with the addition of hydrogen bonds and an electrostatic interaction between Glu85 and Lys97.

MouR-DNA Complex

The GntR superfamily of dimeric transcription factors is characterized by the presence of an N-terminal winged-helix DNA-binding domain. Analysis of electrostatic properties of the winged-helix domain of MouR (Figure 4C) strongly suggested that two residues (Arg44 and Arg48) are involved in the binding to dsDNA. Furthermore, the analysis of the structure of a complex between FadR from *E. coli* and dsDNA (Xu et al., 2001) revealed that these two arginine residues, very conserved in the GntR superfamily (Figure 4D and 1B), are indeed involved in the binding of dsDNA. Based on the superposition of the FadR-dsDNA complex (PDB code 1HW2) onto the winged-helix domain of MouR, we modelled the MouR-dsDNA complex, as shown in the Figure 4A. As expected, Arg44 and Arg48 on the α3-helix are oriented ready to interact with dsDNA. Lys49 is also very close to the major groove of the DNA and can also be involved in the interaction (Figure 4B). Although all known structures of winged-helix domain are very identical, their mode of interaction with dsDNA can vary greatly. While the majority of these interactions involve the α3-helix binding in the major groove of the DNA (Gajiwala and Burley, 2000), our model of the MouR-dsDNA complex (Figure 4A) shows that only the top of this helix is implicated in the interaction with dsDNA.

MouR positively regulates the expression of the *agr* locus by binding to the operon promoter

Based on the MouR structure analysis, we hypothesized that the α3-helix and in particular the conserved Arg44 and Arg48 residues would be needed for its ability to bind target DNA (Figure 3 and 4) and, thus, crucial for its activity as a transcriptional factor. We engineered an *Lm* strain to express, on a Δ*mouR* background, a mutated version of MouR where Arg44 and Arg48 were replaced by alanine residues (*Lm* Δ*mouR*+*mouR*(R44/48A)) and analyzed by RT-qPCR the expression of MouR-regulated genes in the WT, Δ*mouR,* Δ*mouR*+*mouR* and Δ*mouR*+*mouR*(R44/48A) strains. We observed that in the Δ*mouR* strain the expression of the four *agr* genes was highly repressed while *lhrA* was upregulated as detected by RNAseq. In the Δ*mouR*+*mouR* complemented strain the expression of all five genes was restored back to levels comparable to those of the WT (Figure 5A), definitively demonstrating the role of MouR in the expression of the *agr* locus and *lhrA*. Inversely, the Δ*mouR*+*mouR*(R44/48A) strain exhibited expression levels of *agr* genes similar to those observed for Δ*mouR* (Figure 5A). This strongly suggested that indeed MouR contains a DNA-binding site at the N-terminal that when lightly mutated renders it unable to transcriptionally activate *agr* genes.

To investigate whether MouR controls the expression of the *agr* locus by direct activation of the operon promoter, we performed electrophoretic mobility shift assays (EMSAs) to test

protein-DNA interactions. We observed that increasing amounts of MouR were able to delay the migration of the band corresponding to the *agr* promoter (p*agr*), 0.2 µM of MouR being sufficient to delay the *agr* promoter mobility (Figure 5B). Furthermore, p*agr* mixed with increasing amounts of the mutated MouR(R44/48A) failed to delay the migration, clearly indicating that the regulator otherwise able to bind the *agr* promoter becomes effectively incapable of binding it. As controls, we mixed p*agr* with a purified unrelated regulator (Lmo0443), or mixed MouR with its own promoter region. In neither of these cases we were able to detect a band shift (Figure 5B), demonstrating that MouR acts as a DNA-binding transcription factor with specific affinity to the promoter of the *agr* operon.

MouR regulates chitinase activity and biofilm formation

While the *agr* system has not been deeply characterized in *Lm*, it has been consistently associated with some relevant roles. Namely, it appears to be implicated in the chitinase activity and also in the capacity to develop a biofilm (Paspaliari et al., 2014; Riedel et al., 2009). We thus analyzed the potential connection between MouR and these specific phenomena. We first confirmed that the *in vitro* growth of the WT, Δ*mouR* and Δ*mouR*+*mouR* strains were comparable (Figure 6A). To test the chitinase activity of *Lm,* we spotted overnight cultures of the different strains on LB agar plates supplemented with chitin. The formation of a translucent halo on the opaque medium directly correlated with the chitinase activity of the bacteria (Paspaliari et al., 2014). Our data showed that the mutant Δ*mouR* had a striking reduction of chitin hydrolysis when compared with both the WT and Δ*mouR*+*mouR* strains which, in turn, were comparable to each other (Figure 6B). A deletion mutant (Δ*agrC*) for the *agrC* sensor kinase component of the *agr* system (Pöntinen et al., 2015) showed undetectable chitin hydrolysis (Figure 6B). We also investigated the roles of MouR in biofilm formation on PVC microplates by the crystal violet trubidimetry method (Christensen et al., 1985). We observed that, whereas Δ*mouR* developed a significantly reduced biofilm comparing to the WT strain, gene complementation successfully restored the ability to develop a normal biofilm (Figure 6C). Once more, the behavior of Δ*agrC* further showed that the lack of a functional *agr* system renders *Lm* incapable of forming a wild type biofilm, thus confirming the relationship between its control by MouR and biofilm formation.

Altogether, these results showed how, by directly regulating the Agr system, MouR controls chitinase activity and biofilm formation.

MouR is required for cell invasion and virulence

Taking into account the upregulation of *mouR* while *Lm* is infecting the mouse host and its roles in chitinase activity and biofilm formation, we hypothesized that MouR could be important for the invasion of eukaryotic cells and establishment of systemic infection. To test this hypothesis, we infected the human cell lines Caco-2, Jeg-3 and HeLa with *Lm* WT and Δ*mouR* strains and assessed numbers of intracellular bacteria 90 min post-infection. As compared to the WT strain, the Δ*mouR* mutant showed a slight defect in the invasion of all cell lines, especially in the case of Jeg-3 and HeLa cells (Figure 7A), suggesting a role for MouR in cell invasion.

To investigate the role of MouR *in vivo,* female BALB/c mice were intravenously or orally infected with WT, Δ*mouR* or Δ*mouR*+*mouR* strains and the CFUs per liver and spleen were assessed 72 h post-infection (Figure 7B and 7C). While no significant differences were detected in the bacterial burden of intravenously infected animals (Figure 7B), a significant reduction in the number of Δ*mouR* bacteria was observed in the liver of orally infected animals (Figure 7C). Furthermore, in mice infected with the Δ*mouR*+*mouR* complemented strain, bacteria levels in mouse organs were comparable to those observed with WT, revealing a restored phenotype (Figure 7C). Altogether, our data indicate that MouR is necessary for *Lm* virulence in the mouse model.

Discussion

Virulence regulators are crucial elements of the genome of pathogenic bacteria (Camejo et al., 2011). They coordinate the transcriptional shift that mediates the switch from a saprophyte into a pathogen, granting the adaptability necessary to infect host organisms (Camejo et al., 2009; Cossart and Toledo-Arana, 2008). However, despite the high abundance of transcription factors in bacterial genomes, proper characterization and data on their 3D structures is not very abundant (Rodionov, 2007; Sai et al., 2011). It has been predicted that at least 209 transcription regulator-coding genes exist in the genome of *Lm* EGD-e (Glaser et al., 2001). Whereas PrfA occupies the highest seat in the virulence regulation of *Lm*, controlling the expression of most of the main virulence factors (Freitag et al., 2009), the identification of novel regulators is key to understand how *Lm* fine tunes the expression of virulence determinants in order to optimally promote virulence (Freitag et al., 2009). Here, we report the discovery and molecular characterization of a novel virulence regulator MouR along with the demonstration of its roles in the *Lm* virulence regulatory network as the transcriptional activator of the Agr system.

To our knowledge, this is the first time a regulator of the Agr system of *Lm* is identified. At least a dozen regulators have been linked to the regulation of *agr* in *S. aureus*, with some exerting positive control and others acting as repressors (Thoendel et al., 2011). Although we do not rule out the possibility of existence of other regulators with control over Agr in *Lm* and also the possibility of a system autoregulation as suggested before (Riedel et al., 2009; Rieu et al., 2007), our data strongly indicate that MouR has a predominant role as the central activator of *agr*.

Transcription factors can control gene expression in a direct or more indirect fashion. This holds true in the case of *S. aureus* Agr in which some of its regulators directly bind the *agr* promoter, while others seem to have an indirect mode of control (Thoendel et al., 2011). Our results not only show the specific binding of MouR to the promoter region of the *agr* locus but also demonstrate that the mutation of the DNA-binding site eliminated such interaction, suggesting that *Lm* has evolved a regulatory system to specifically control the Agr system. In addition, the incapacity of MouR to bind to its own promoter suggests the absence of MouR auto-regulation.

By resolving the 3D structure of MouR we opened ways to the classification and deeper characterization of this novel regulator. As initially predicted by bioinformatics, the crystal structure of MouR revealed that a monomer is composed of a typically conserved GntR N-terminal with a winged-HTH DNA-binding domain and a more variable C-terminal with an E-O domain. Through deeper analysis of the C-terminal structure, we have classified MouR as a

member of the FadR sub-family, the largest sub-group of the GntR superfamily (Suvorova et al., 2015). The presence of exactly six α-helices within the C-terminal allowed us to further classify it as a member of the VanR sub-class (Jain, 2015). Despite the large size of the FadR sub-family, currently making up close to 50% of all GntR proteins sequences on Pfam, structural data of only five VanR regulators were available on Pdb at the time of this study (Jain, 2015; Lord et al., 2014; Zheng et al., 2009). To our knowledge this is also the first VanR type regulator to be described in *Listeria*. As already reported for GntR transcription factors that predominantly bind their DNA-targets as dimers (Jain, 2015; Rigali et al., 2002), we demonstrated that MouR organizes and functions as a dimeric unit. However, the quaternary dimer structures among this family as well as how they interact with DNA, can differ. We reveal that the MouR dimer has a head-to-head orientation and how the α3-helices solely interact with the DNA through the Arg44 and Arg48 residues.

The Agr system was previously shown to have an impact on both *Lm* stress response/adaptation and pathogenicity, being in particular solidly linked to chitinase activity, biofilm formation and virulence (Riedel et al., 2009). The Agr system promotes chitinase activity through negative regulation of the sRNA *lhrA* which itself binds to the mRNA of the chitinase-coding gene *chiA* blocking ribosome access and preventing translation (Figure 8) (Nielsen et al., 2011; Paspaliari et al., 2014). Furthermore, *Lm* chitinase has been established as a virulence factor with roles in pathogenicity related to modulation of the immune system, through suppression of the hostile inducible nitric oxide synthase (iNOS) (Chaudhuri et al., 2013; Frederiksen et al., 2013). Given the dual function of its chitinase, *Lm* appears to improve the adaptation to both the environment and the host through MouR. This could be a factor contributing to the prevalence of a conserved *mouR* locus in pathogenic and non-pathogenic *Listeria* strains.

We also demonstrate here that MouR plays a role in the formation of *Lm* biofilm on abiotic surfaces. The relevance of biofilm formation in the context of *Lm* infection is not fully understood but it is a common general key feature for pathogen adaptation both outside and inside the host (Jamal et al., 2017). Once established on surfaces or tools, biofilms drastically increases *Lm* survival, persistence and ultimately promote dissemination (Colagiorgi et al., 2017; Langsrud et al., 2004). Even if its role in biofilm formation may vary depending on several conditions, the dependence of this phenomenon on Agr is clear (Garmyn et al., 2012; Rieu et al., 2007, 2008). Our data show that deletion of *mouR* does compromise biofilm formation to a similar extent of that of a Δ*agrC* mutant. The regulation of biofilm formation in bacteria is very complex and in *Lm* Agr is only one of its several regulatory elements, like the biofilm-promoting motility proteins flagellins (Vatanyoopaisarn et al., 2000) or the biofilm-repressing *luxS* signal transduction pathway (Sela et al., 2006). The orphan response regulator DegU has also been

shown to play a role in Lm biofilm formation (Gueriri et al, 2008), partly independent of its control over flagella-related genes. In addition, a transcriptomics study revealed that DegU could positively control *mouR* expression at 24°C (Williams et al., 2005). DegU might thus have some transcription control over mouR and, that way, adding another layer to the Agr-biofilm regulatory scene.

We show here that MouR appears to play a role in cell invasion. Previous studies have demonstrated that the expression of internalins InlA and InlB as well as InlA allocation to the membrane surface are decreased in absence of a functional Agr system (Garmyn et al., 2012; Riedel et al., 2009), inducing a defect of cell invasion (Riedel et al., 2009; Zetzmann et al., 2016). Even if we did not observe a significant downregulation of *inlA/B* expression in absence of MouR, the invasion defect observed for the *mouR* mutant could be related to a slight deregulation of *Lm* internalins due to impaired Agr functionality.

Importantly, we demonstrated that MouR is necessary for full *Lm* virulence in the mouse model, in particular following oral inoculation. The absence of a defect in infectivity after intravenous inoculation points towards a role for MouR during the gastric phase of infection. The impaired ability to develop a biofilm in absence of MouR could possibly impact *Lm* prevalence in the digestive tract and the success of crossing the intestinal barrier, as previously proposed (Begley et al., 2009). We also observed that the significant decreased infectivity of the *mouR* mutant is almost only detected in mouse livers. A similar phenotype has been reported for a Δ*agrD* mutant showed by bioluminescence to spread to the spleen but not the liver of infected mice (Riedel et al., 2009). Considering the role of MouR and Agr on chitinase function, a differential modulation of iNOS/NO based immune response between organs could account for such differences. In this sense, an endotoxic shock induces distinct levels of iNOS expression in the spleen and the liver (Kan et al., 2004). Further studies could reveal a role for MouR/Agr in the differential modulation of the immune response between organs and/or cell types.

We thus propose MouR as a dimeric DNA-binding transcription factor expressed by *Lm* to regulate, through the Agr system, biofilm formation and control the host immune response to promote bacterial virulence. This work points out MouR as target for innovative strategies against *Lm* biofilm formation.

Acknowledgments

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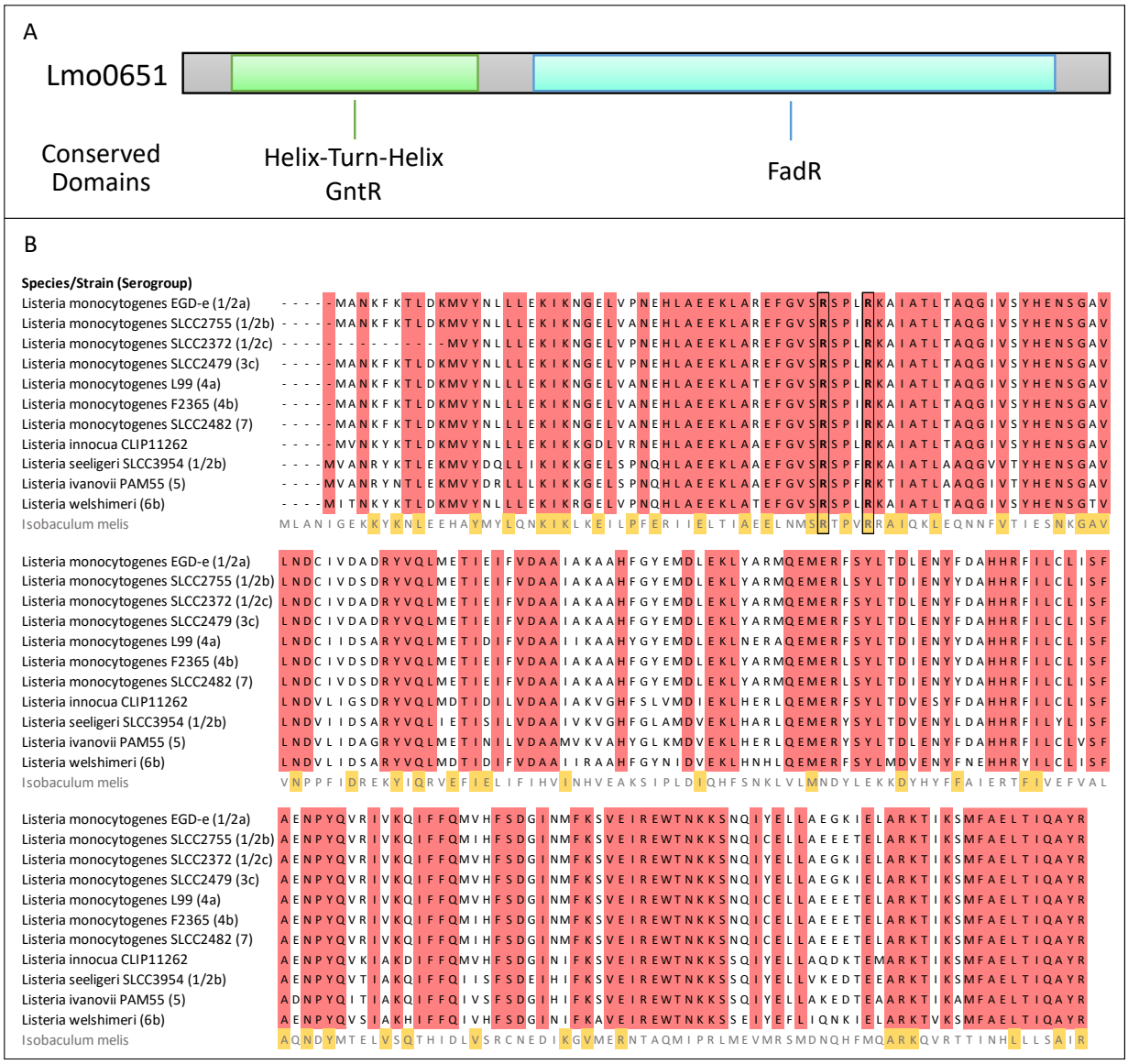

Figure 1. *lmo0651* encodes a novel GntR transcription factor. **(A)** Bioinformatic analysis predicts the locus *lmo0651* of *Lm* to encode a transcription factor with a typical DNA-binding winged helix-turn-helix GntR domain and a FadR domain. **(B)** Alignment of protein sequence of Lmo0651 from different *Listeria* species with conserved sites highlighted in red. The sequence of the closest relative outside of the *Listeria* genus found by BLAST analysis (*Isobaculum melis* - putative GntR transcriptional regulator) is marked in yellow for conserved site after alignment against *Lm* EGD-e Lmo0651. Critical DNA-binding arginines are highlighted in bold.

A

Gene	Fold change†
agrD ‡	- 144,19
agrB	- 77,76
agrC	- 29,15
agrA	- 20,92
fruA	- 5,04
lmo0278	- 3,87
lhrA	+ 7,32

† Fold change values refer to expression of genes significantly regulated in *Lm* Δ*mouR* as shown by *in vitro* transcriptome vs. WT.

‡ Gene did not make statistical stringency cutoff.

B

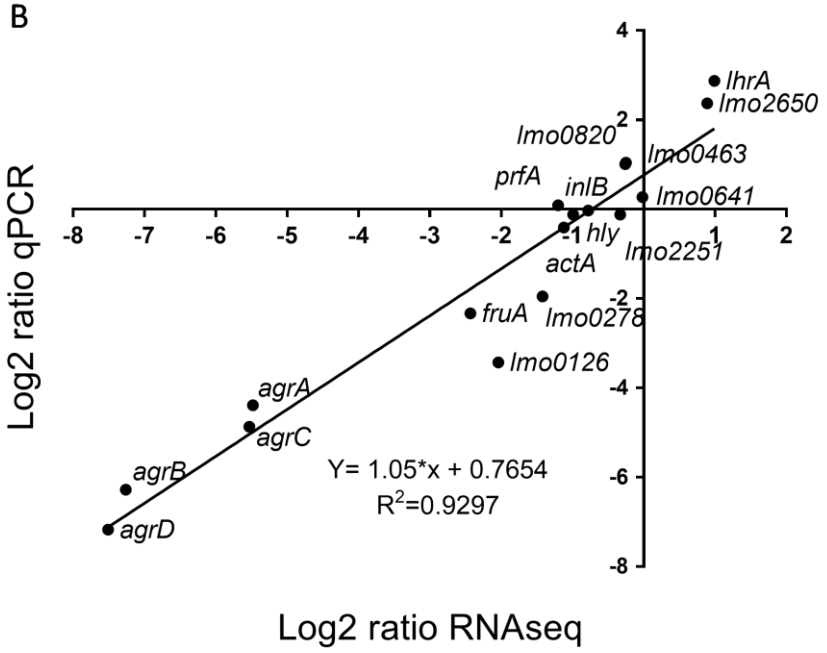

Figure 2. Genes differently regulated in Δ*mouR in vitro.* **(A)** Fold change of the expression of genes regulated by MouR as shown by RNAseq. **(B)** Validation of RNAseq transcriptome data by RT-qPCR. Data represented as Log2 of fold change between *in vitro* gene expression of *Lm* WT and Δ*mouR* in exponential growth in BHI at 37°C as measured by RNAseq and RT-qPCR.

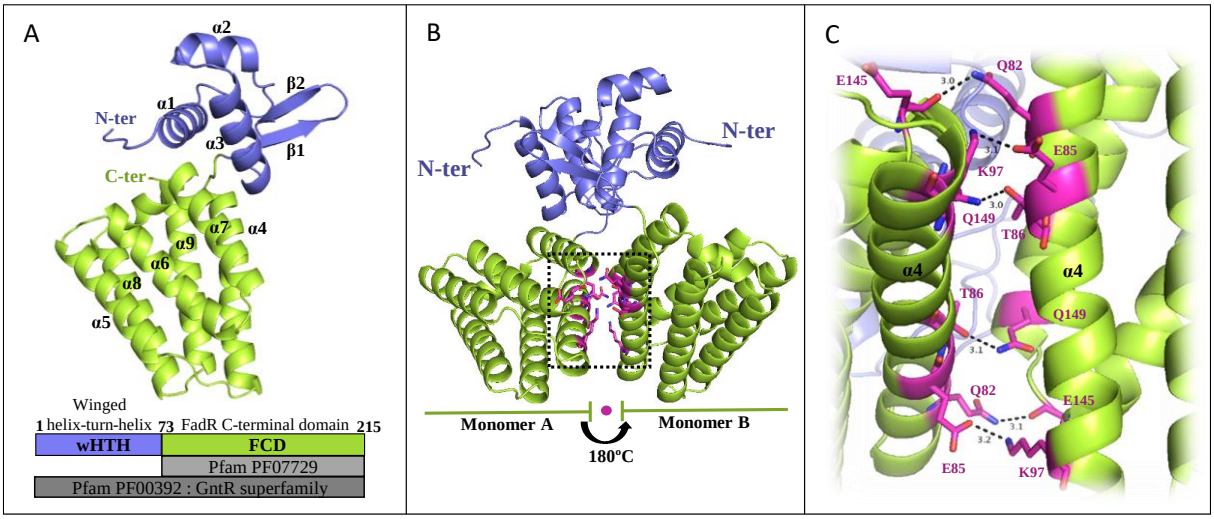

Figure 3. MouR 3D structure and dimerization interface. **(A)** Top: ribbon presentation of the MouR 3D structure. The N- and C-termini and secondary structure elements are indicated. Color code: blue, winged HTH domain; green, FCD domain. Bottom: linear representation of MouR structural domains according to Pfam (color code as the top). **(B)** Ribbon representation of the 3D structure of MouR dimer. **(C)** The C-terminal dimerization zone is enlarged to show some of the residues (represented as magenta sticks) involved in the interaction. Hydrogen bonds are represented by dashed lines, and distances between residues are indicated in Å.

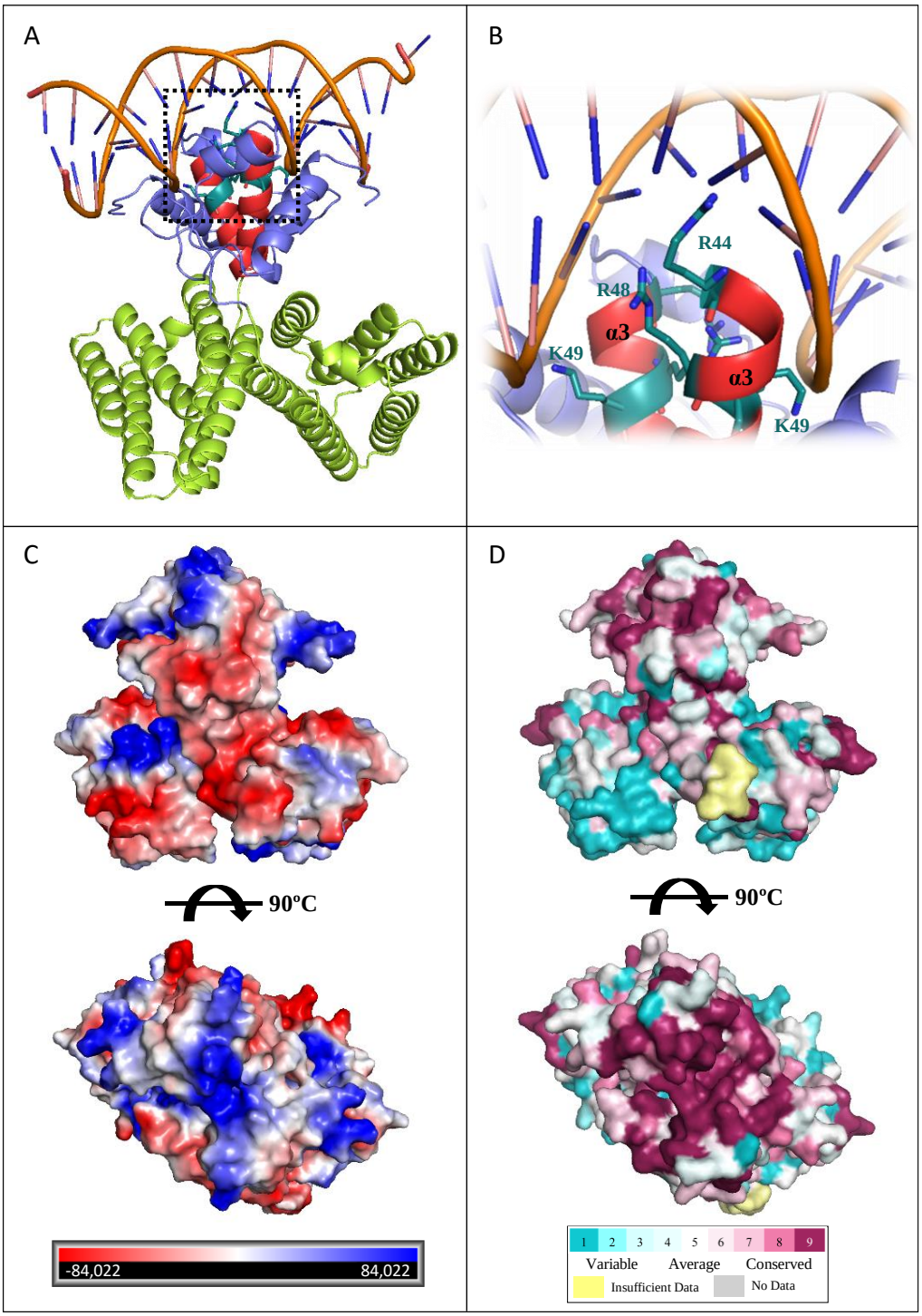

Figure 4. Model of MouR-dsDNA complex. **(A)** Ribbon representation of the modelled MouR-DNA complex. The DNA is modeled into this figure based on the superposition of the FadR-DNA complex (PDB ID: 1HW2) onto the winged-HTH domain of MouR. **(B)** The overall architecture of the winged-HTH domain of MouR (shown in blue), with putative DNA-binding residues shown (represented as sticks). The helix (α3) involved in the interaction with DNA is colored in red. **(C)** The electrostatic potential surface of MouR. The potential is given with the negative (red) and positive (blue) contour level in the range -84.0 to +84.0 kBT, respectively. **(D)** Sequence conservation projected onto the surface as reported by ConSurf (http://consurf.tau.ac.il/2016/). The least conserved residues and highest conserved residues are colored from cyan to dark purple. All structures representations are rendered in the same orientation.

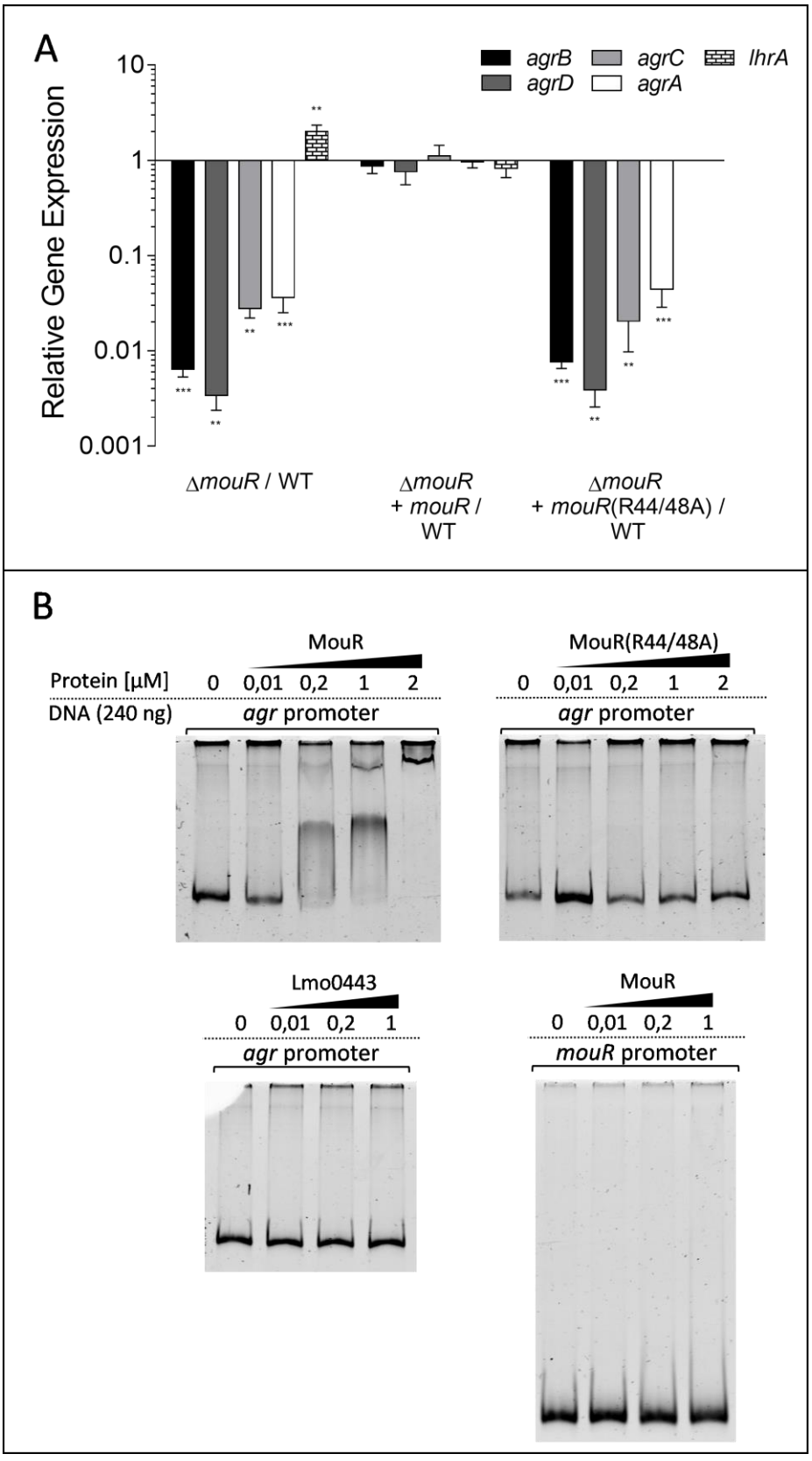

Figure 5. MouR positively regulates the expression of the *agr* locus by directly binding to the operon promoter. **(A)** Expression of *agr* genes and the sRNA *lhrA* in standard growth conditions. RT-qPCR performed with total RNA isolated from *Lm* WT, Δ*mouR*, Δ*mouR*+*mouR*

or Δ*mouR*+*mouR*(R44/48A) cultures at an exponential growth in BHI medium at 37°C. Expression levels are represented relative to WT. Values are mean ± SD of three independent experiments. **, $p \leq 0.01$; ***, $p \leq 0.001$. **(B)** Electrophoretic mobility shift assays on polyacrylamide gels of increasing amounts of purified MouR or MouR(R44/48A) with a DNA fragment containing the promoter region of *agr* or increasing amounts of an unrelated putative regulator (Lmo0443) with the same DNA and increasing amounts of MouR with an unrelated DNA (promoter region of *mouR*). Results are representative of at least 3 independent experiments.

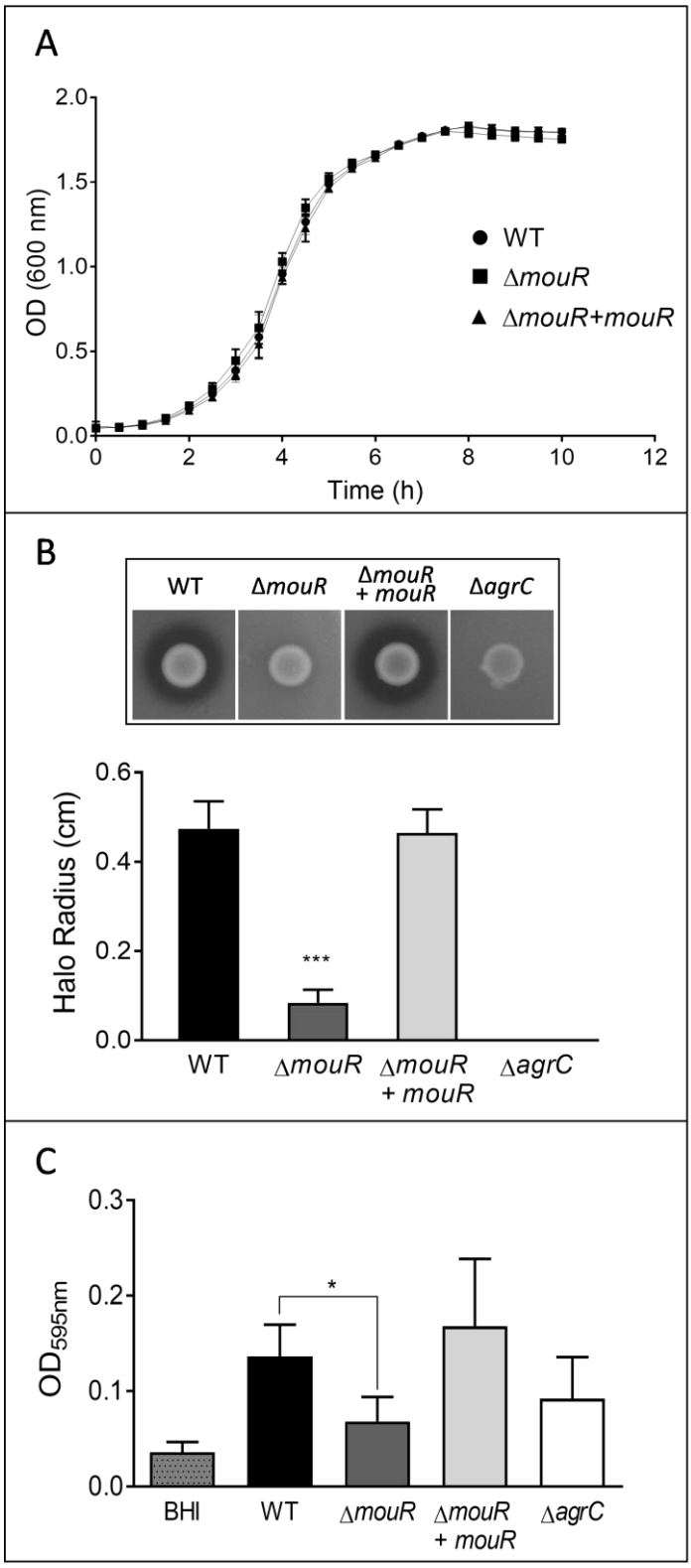

Figure 6. MouR regulates chitinase activity and biofilm formation. **(A)** Growth curves of different *Lm* strains in BHI. Overnight cultures were diluted 100-fold in BHI medium and incubated at 37°C with agitation. Measurement of optical density (OD_{600nm}) was performed every 30 min. **(B)** Chitinase activity of *Lm* WT, Δ*mouR*, Δ*mouR*+*mouR* and Δ*agrC*. Overnight cultures were spotted on LB agar plates supplemented with chitin and incubated at 30°C for 6

days. Halo measurements are mean ± SD of four independent experiments. **(C)** Biofilm formation of WT, Δ*mouR*, Δ*mouR*+*mouR* and Δ*agrC*. Cultures were diluted 100-fold in BHI, incubated in 96-well microplates for 20 hours and biofilm formation was measured by the crystal violet assay. Wells with BHI medium served as control. Results are mean ± SD of three independent experiments. *, $p \leq 0.05$; ***, $p \leq 0.001$.

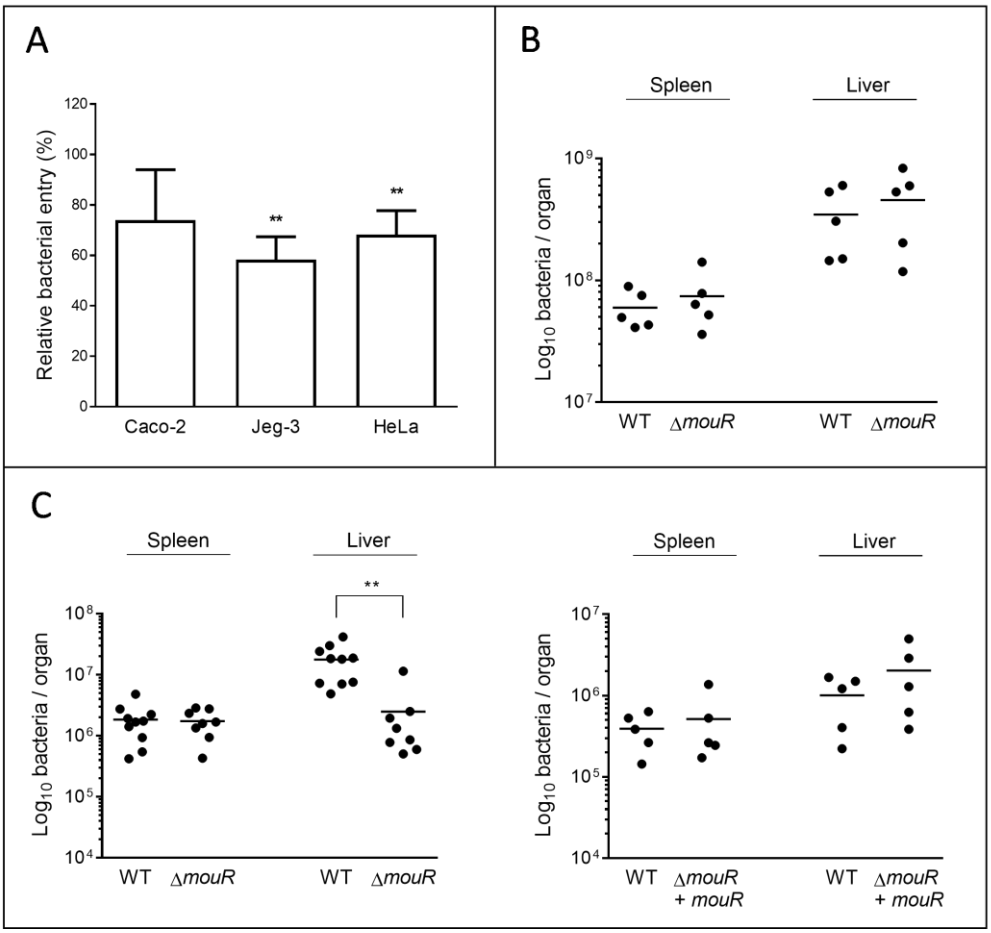

Figure 7. MouR is required for cell invasion and virulence. **(A)** Invasion of *Lm* WT and Δ*mouR* into Caco-2, Jeg-3 and HeLa cell monolayers, shown as intracellular CFU counts relative to WT (fixed at 100%). Results are mean ± SD of three independent experiments. CFU counts in spleens and livers of female BALB/c mice 72 h after **(B)** intravenous infection with 10^4 CFU of WT or Δ*mouR* and **(C)** oral infection with 10^9 CFU of WT, Δ*mouR* or Δ*mouR*+*mouR*. Each dot of the plot corresponds to one animal, mean values are represented by a horizontal bar. **, $p \leq 0.01$.

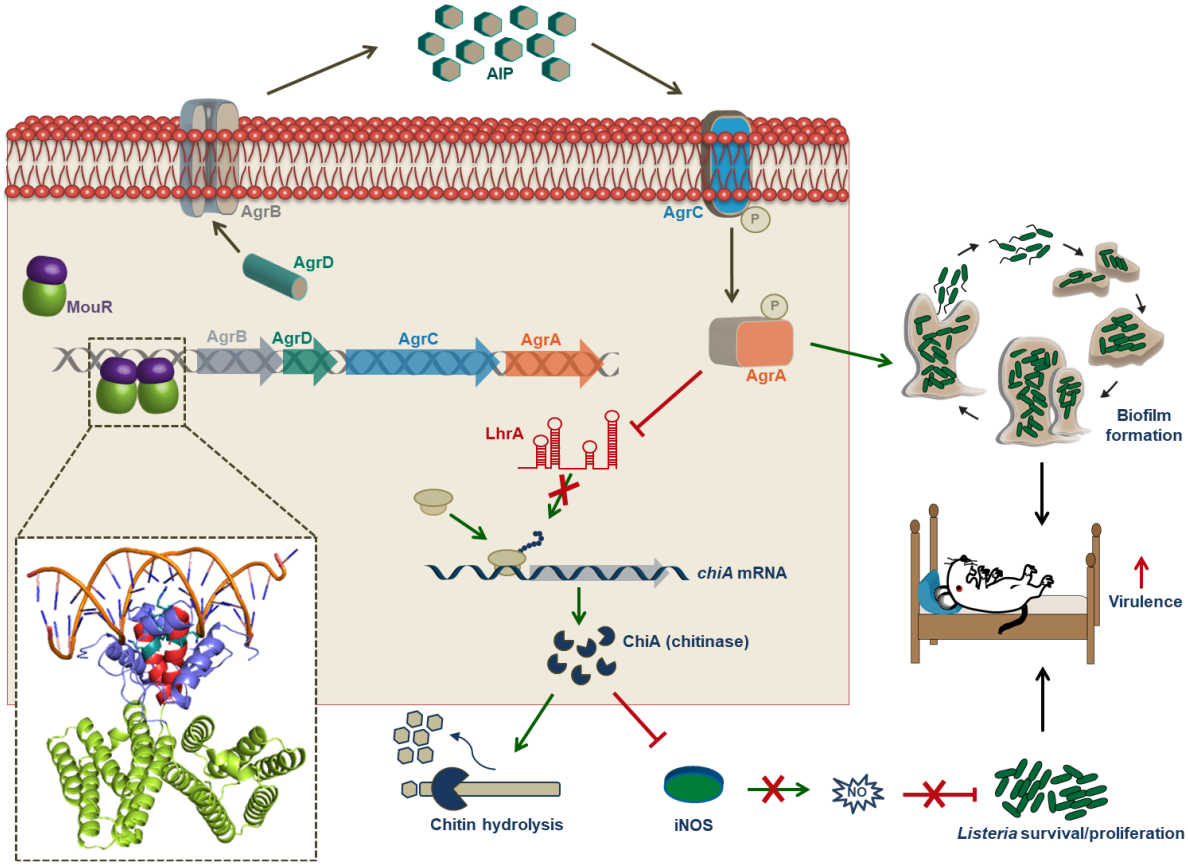

Figure 8. MouR controls the expression of the *Listeria monocytogenes* Agr system and mediates virulence.

Table 1 - Plasmids and bacterial strains

Plasmid/Strain	Description	Source
Plasmids		
pMAD	Gram-negative/Gram-positive shuttle vector; thermosensitive replication; Amp^r Ery^r	Arnaud et al., 2004
pMAD(Δ*mouR*)	pMAD with 5'- and 3'-flanking regions of *mouR* locus; Amp^r Ery^r	This study
pIMK	*L. monocytogenes* phage-derived site-specific integration vector; Kan^r	Monk et al., 2008
pIMK(*mouR*)	pIMK with *mouR* locus and 5'- and 3'-flanking regions; Kan^r	This study
pIMK(*mouR*-Mut)	pIMK with *mouR* locus mutated at R44A + R48A and 5'- and 3'-flanking regions; Kan^r	This study
pET28	Vector system for cloning and expression of recombinant proteins in *E. coli*	Novagen
pET28a(*mouR-6His*)	pET28a with *mouR* locus fused with a 6-Histidin tag at C-Terminal; Kan^r	This study
pET28a(*mouR*- Mut)	pET28a with *mouR* locus mutated at R44A + R48A and fused with a 6-Histidin tag at C-Terminal; Kan^r	This study
E. coli		
DH5α	Cloning host strain; F^- Φ80*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Life Technologies
BL21(DE3)	F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)	Invitrogen
Listeria monocytogenes		
EGD-e	Wild type; serotype 1/2a	Glaser et al., 2001
EGD-e Δ*mouR*	EGD-e *mouR* deletion mutant	This study
EGD-e Δ*mouR + mouR*	EGD-e *mouR* deletion mutant complemented with pIMK(*mouR*); Kan^r	This study
EGD-e Δ*mouR + mouR* (R44/48A)	EGD-e *mouR* deletion mutant complemented with pIMK(*mouR*-Mut); Kan^r	This study
EGD-e Δ*agrC*	EGD-e *agrC* deletion mutant	Pöntinen et al., 2015

Table 2 - Data collection and refinement statistics of MouR. The numbers in parentheses are for the highest resolution shell

	SeMet (SAD dataset)	Native
Data Collection		
Space Group	$P4_1$	$P4_1$
Wavelength (Å)	0.97915	0.980065
Cell dimensions		
a,b,c (Å)	121.415, 121.415, 60.614	122.445, 122.445, 61.13
α,β,γ (º)	90, 90, 90	90, 90, 90
Resolution (Å)	42.89 - 2.793 (2.893 - 2.793)	43.29 - 2.2 (2.279 - 2.2)
Number of observations measured	73122 (7408)	247605 (25155)
Number of unique reflections measured	22034 (2175)	46113 (4603)
Multiplicity	3.3 (3.4)	5.4 (5.5)
Completeness (%)	99.06 (96.80)	99.30 (96.92)
I/σI	10.05 (1.43)	8.62 (0.58)
R_{merge}	0.1029 (0.8352)	0.1159 (1.878)
CC (1/2) (%)	99.6 (59.5)	99.8 (44.4)
Refinement		
R_{work}/R_{free} (%)		22 / 24.84
Total atoms		3732
Average B (A^2)		72.05
RMSD from standard stereochemistry		
Bond lengths (Å)		0.003
Bond angles (º)		0.56
Ramachadran plot statistics		
Favored (%)		97.9
Allowed (%)		2.1
Disallowed (%)		0
PDB Code		**6EP3**

Table S1 - Primers

Name	Sequence (5' - 3')
EMSA	
EMSA-*agr*-F	AGACCTTAGATCATGTAAACC
EMSA-*agr*-R	TTTCGAAATCAACACATCCG
EMSA-*mouR*-F	ATCTATCGGATTAATGCAGG
EMSA-*mouR*-R	CATTCGGCACTAACTCACC
*Plasmid/mutant construction**	
mouR- A	ATTGTCGACCGGCCAACTTCATTCGCG
mouR- B	ATCCATGGCACAAAGTCTGCCCCCTCTATGC
mouR- C	CGCCATGGTAAAAAAGCTGTCACTTTTGTGG
mouR- D	CTGAGATCTATTCCTCTTGCTGTAGCG
mouR- F	GATGCGGATCGTTATGTGC
mouR- R	GACGATACGCACTTGATAGGG
pIMK-*mouR*-F	ACGTCTGCAGTGTCGGAAAAGGGATTGC
pIMK-*mouR*-R	GGCAGTCGACGATTGGGATACAGATTTGTGG
mouR (pET28)-F	ACGCCCATGGCAAACAAATTTAAAACATTAGAT
mouR (pET28)-R	TCCGCTCGAGTCTGTATGCTTGAATCGTT
mouR- R44/R48->A44/A48-F	GAGAATTCGGTGTTAGCGCCTCACCACTCGCAAAAGCCATTGCAACGC
mouR- R44/R48->A44/A48-R	GCGTTGCAATGGCAAAAGCGAGTGGTGAGGCGCTAACACCGAATTCTC
lmo0443 (pET28)-F	ACAACCATGGCAAGACATGCACAAAAAAG
lmo0443 (pET28)-R	AACCCTCGAGCTTTAGAAAATCTTGTAATGCTT
PL95	ACATAATCAGTCCAAAGTAGATGC
PL102	TATCAGACCTAACCCAAACCTTCC
RT-qPCR	
qPCR-*16S*-F	GCGTAGATATGTGGAGGAAC
qPCR-*16S*-R	CAGGCGGAGTGCTTAATG
qPCR-*actA*-F	CGAGCCTACCAGTAATCC
qPCR-*actA*-R	CTGATTCGCTTTCCTCTACC
qPCR-*agrA*-F	GCCTACACATCAAGGTATGG
qPCR-*agrA*-R	ACTTCCGAATTTCCTGAGC
qPCR-*agrB*-F	TCAGAAAGAATGGCGGATG
qPCR-*agrB*-R	CCTGTTACTAAGGCGATACC
qPCR-*agrC*-F	AGAAGGTCGTGGATTAGG
qPCR-*agrC*-R	TCTCTATCGGTCACTTTCG
qPCR-*agrD*-F	AGAAGAACAATCCATGAAAGTTGC
qPCR-*agrD*-R	TTCTTGCATTTTCACAAATGGACT
qPCR-*fruA*-F	TTGTAATTGGTGGAGGTATCG
qPCR-*fruA*-R	GCGAGTTGATCTTGAGGAA
qPCR-*inlB*-F	AGCACAACCCAAGAAGGA
qPCR-*inlB*-R	GGCACGGTGATAGTCTCC
qPCR-*hly*-F	TCGTCCATCTATTTGCCAGG
qPCR-*hly*-R	TTACCGTTCTCCACCATTCC
qPCR-*lhrA*-F	CGTGTAGTTCATGTCTATGC
qPCR-*lhrA*-R	ATAAACATTTCCAGCGTTGC
qPCR-*lmo0126*-F	GTTAGTGTGAATGGTAAGG
qPCR-*lmo0126*-R	ATCCGTAGTGTATGTTGC
qPCR-*lmo0278*-F	TGATGAGCCAATCGTTATCG
qPCR-*lmo0278*-R	CATAAATTCAGCACCAGTAAGC
qPCR-*lmo0463*-F	GAGGTGTTCATTTCAGAGG
qPCR-*lmo0463*-R	TAAGGACGAGCACTAAGC
qPCR-*lmo0641*-F	CAATTCAAGCCGAACTAGG
qPCR-*lmo0641*-R	CACACTGCCATAAGTAACC
qPCR-*lmo0820*-F	GGCATCAGATACGAAGAACCAAT
qPCR-*lmo0820*-R	TTCCAACACCATGACCACGATA
qPCR-*lmo2251*-F	GAGAAGTGGTCGTAGTTATCG
qPCR-*lmo2251*-R	GGTTTGTTTCGCCATTAGG
qPCR-*lmo2650*-F	CTCGCTCAATGAATGTGGAT
qPCR-*lmo2650*-R	TTGTTGACGATGACTACTTTGG
qPCR-*prfA*-F	CGAGTATTAGCGAGAACG
qPCR-*prfA*-R	GATAACGTATGCGGTAGC

* Restriction sites are underlined

Table S2 - Accessibility of residues involved in dimerization of MouR. Type of interaction: Hph, hydrophobic Hyd, hydrogen bond; Sal, salt bridge. Accessibility: ASA, accessible surface area (Å2) in the monomer; BSA, buried surface area (Å2), i.e., surface engaged in the dimerization interface.

Residue	Location	Interact with	Type	Distance (Å)	ASA	BSA
Q82	α4	E145 - N146	Hyd	2.96 - 3.25	112.72	72.55
E85	α4	K97	Sal	3.13	95.9	42.66
T86	α4	N146 - Q149	Hyd	3.87 - 3.08	44.53	41.42
I89	α4	I89 - A93	Hph	4.0 - 3.9	112.2	77.83
F90	α4	F90	Hph	3.5	35.63	35.51
A93	α4	I89	Hph	3.6	54.35	30.7
K97	α4	E85	Sal	3.19	70.15	29.75
E145	Loop between α6-α7	Q82	Hyd	3.14	106.94	15.46
N146		Q82 - T86	Hyd	3.39 - 3.72	40.89	38.97
Y148	α7	Q159	Hyd	3.76	152.67	132.97
Q149	α7	T86	Hyd	3.07	38.57	34.51
I152	α7	I152	Hph	3.7	81.83	55.9

PART II

Identification and characterization of Lmo0443, a novel *Listeria monocytogenes* virulence regulator

Introduction

The switch that occurs in gene expression when a pathogen goes from the environment into the host is a crucial mechanism to ensure survival, multiplication and spreading. Hence, pathogenic bacteria must evolve complex but well-coordinated regulatory mechanisms in order to fine tune the expression of their genomes, in particular their virulence factors-encoding genes (Freitag et al., 2009). Continuous identification and characterization of components of these virulence regulatory networks is very important for the elucidation of new molecular mechanisms presented by pathogenic bacteria, for a better understanding of host response mechanisms and even for the contribution to the evolution of diagnosis and therapy options (Cossart and Lebreton, 2014; Radoshevich and Cossart, 2017). Transcription factors are one of the major components of the gene expression regulation machinery and play fundamental roles in virulence. Whereas in *Lm* the transcription factor PrfA has a fundamental role in activating the transcription of many of the major virulence factors implicated in the process of eukaryotic cell infection (e.g. LIPI-1 locus, *inlA/inlB* locus, etc.) (de las Heras et al., 2011), many other transcription factors with roles in *Lm* virulence have been and are currently being discovered (Christiansen et al., 2004; Kamp and Higgins, 2009; Mandin et al., 2005; Mauder et al., 2008; Oliver et al., 2010; Shen and Higgins, 2006). Here we attempt to characterize Lmo0443, a putative LytR transcription regulator of *Lm* that we recently identified as upregulated during *Lm* infection of mice spleen as compared to growth in BHI.

The LytR family of transcription factors belongs to the larger group of LytR-CpsA-Psr (LCP) proteins. The LCP family is a group of cell-envelope associated putative transmembrane transcription attenuators that are unique to bacteria and contain a LytR-CpsA-Psr motif, which is predicted to be extracellular (Hübscher et al., 2008). The first identification of the LytR regulator was in *Bacillus subtilis* where it was described to repress the expression of both the bacterial autolysis associated *lytABC* transcript, which encodes the putative lipoprotein LytA, the N-acetylmuramoyl-L-alanine amidase LytC and its modifier LytB, and also LytR itself (Lazarevic et al., 1992). LCP proteins are found throughout Gram-positive bacteria but are rare in the Gram-negative group (Hübscher et al., 2008). This family then gained increasing popularity when roles of LCP members in regulation of virulence factors, antibiotic resistance and in cell envelope maintenance were described (Chatfield et al., 2005; Cieslewicz et al., 2001; Rossi et al., 2003; Wang et al., 2015; Wen et al., 2006). Evidence have also indicated that LytR and other LCP proteins are capable of alternative roles such as to enzymatically catalyze the attachment of wall teichoic acids (WTA) to peptidoglycan (Gale et al., 2017; Kawai et al., 2011). Nonetheless, the precise role of the LytR-CpsA-Psr motif still requires clarification and the identification and characterization of newly occurring LCP in pathogenic bacteria will further elucidate its roles in virulence (Hübscher et al., 2008).

A cluster of genes was identified in *Lm* as the stress survival islet (SSI-1). In *Lm* EGD-e this islet is composed of five genes, *lmo0444, lmo0445, lmo0446* (*pva*) *lmo0447* (*gadD1*)*, and lmo0448* (*gadT1*) and although it is only found in some *Lm* strains, it is flanked by two genes, *lmo0443* and *lmo0449*, which are very conserved across the *Listeria* genus (Hein et al., 2011; Ryan et al., 2010). A recent screening of 117 *Lm* strains detected the presence of SSI-1 in 32.5% of the strains, which belong mostly to the 1/2c, 3b, and 3c serotypes (Hein et al., 2011). Studies using mutants for components of SSI-1 have revealed roles of this island in stress resistance like tolerance to bile, acidic pH and high salt (Begley et al., 2005; Cotter et al., 2005; Ryan et al., 2010). Recently, a second stress survival islet 2 (ISS-2) with roles in alkaline and oxidative stress resistance has been identified predominantly in the high persistence *Lm* ST121 strains (Harter et al., 2017).

Here we identify Lmo0443 as a putative LCP virulence regulator of *Lm* and evaluate a possible role in the regulation of *lmo0444* from the SSI-1.

Material and methods

Bacterial strains and growth conditions

Strains used are detailed in Table 1. *Lm* EGD-e (ATCC-BAA-679) and *E. coli* were routinely cultured in Brain Heart Infusion (BHI) and Lysogeny Broth (LB) (Difco), respectively. Cultures were grown at 37°C aerobically with shaking. BHI-agar and LB-agar (Difco) plates were used for growth on solid media. To draw growth curves of *Lm* strains, overnight cultures were diluted 1:100 in fresh BHI and absorbance of the culture ($OD_{600\ nm}$) was measured every 30 min. To evaluate the sedimentation of bacteria in liquid culture, bacteria were routinely grown as before in plastic pre-culture tubes until the stationary phase. Tubes were then immobilized in a vertical position for two days. Antibiotics were added to the media whenever appropriated: ampicillin 100 µg/ml, erythromycin 5 µg/ml and kanamycin 50 µg/ml.

Cloning

The deletion of *lmo0443* from the EGD-e wild type strain was achieved by a double homologous recombination process with the suicide plasmid pMAD (Arnaud et al., 2004). The detailed procedure was previously described (Carvalho et al., 2015) and was performed with the primers listed in Table 2. For gene overexpression the coding region of *lmo0444* was cloned in the integrative overexpression plasmid pIKM-2 (Monk et al., 2008), resulting in the vector pIMK-2(*lmo0444*) which was inserted into *Lm* EGD-e chromosome in a single copy.

Bioinformatic analyses

Gene sequences were obtained from the Genbank database (Benson et al., 2013) and homologue searches were performed with the BLAST (Boratyn et al., 2013) tool. Search of conserved protein domains and prediction of protein function were performed with the web-based PROSITE (Sigrist et al., 2013) and NCBI's Conserved Domain Database (Marchler-Bauer et al., 2017) tools.

RNA isolation and RT-qPCR

Lm cultures were grown in BHI to an exponential phase ($OD_{600\ nm}$= 1.0) and total RNA isolation was done by the phenol-chloroform method described elsewhere (Milohanic et al., 2003), with modifications (Pinheiro et al., 2017). After bacteria lysis, isolation was performed with the TripleXtractor reagent (Grisp) following the manufacturer's recommendations. RNA samples

were depleted for DNA by DNase treatment (Turbo DNA-free, Ambion) and RNA purity and integrity was verified by 1% (w/v) agarose gel electrophoresis and Experion Automated Electrophoresis System (Bio-Rad Laboratories) virtual gel analysis. RNAs were reverse-transcribed into cDNA with a random hexamer cocktail-based kit (iScript Kit, Bio-Rad Laboratories) and RT-qPCR was performed using the primers in Table S1 with the iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) and a real-time PCR detection system (iQ5, Bio-Rad Laboratories) with the following cycling protocol: 1 cycle at 95°C (3 min); 40 cycles at 95°C (10 s), 56°C (20 s) and 72°C (20 s). The recorded data was normalized to that of a reference housekeeping gene (16S rRNA) and analyzed by the comparative threshold (ΔΔCt) method.

RNAseq transcriptome

DNA-free total RNAs were depleted for predominant rRNA species by processing with MICROBExpress Bacterial mRNA Enrichment Kit (Ambion) according to the manufacturer's recommendations. Efficient enrichment was verified by Experion Automated Electrophoresis System (Bio-Rad Laboratories) virtual gel analysis and Qubit 3.0 Fluorometer (Thermo Fisher Scientific) analysis. Sequencing of mRNAs was performed using the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) and quality control for RNA fragmentation and library construction was assessed by Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and 2200 TapeStation (Agilent) analyses. Template preparation was achieved with an Ion Chef system (Thermo Fisher Scientific) and sample sequencing was done in triplicates with an Ion Proton System (Thermo Fisher Scientific). Generation of sequence reads and read trimming and filtering was done with Torrent Suite v4.4 software with a FileExporter v4.4 plugin for generation of FASTQ/BAM files. To assess differentially expressed genes between *Lm* WT and Δ*lmo0443* mutant strain, sequencing reads were aligned to the reference genome sequence of *Listeria monocytogenes* EGD-e (RefSeq: GCF_000196035.1, GenBank: GCA_000196035.1, assembly ASM19603v1 -http://www.ncbi.nlm.nih.gov/assembly/GCF_000196035.1) using the aligner TopHat2 (Kim et al., 2013). After transcript assembly, the relative abundance of each transcript was estimated by calculation of the metric fragments per kilobase of transcript per million mapped reads (FPKM) (Trapnell et al., 2011) using Cufflinks and Cuffdiff tools (Trapnell et al., 2013). Statistical significance was attributed to transcripts with fold change of expression higher than 2 or lower than 0.5, a p-value below 0.5 and an FDR-adjusted p-value below 0.1. Genomic alignment and differential expression analysis was performed as a service by the company Bioinf2Bio (www.frombioinformatics2biology.com).

Bright-field microscopy

Lm overnight cultures were diluted 1:100 in BHI and routinely grown in BHI. A small sample from the exponential phase culture ($OD_{600\ nm}$ =1) was directly spread on a glass slide. Bright-field images were collected with an Olympus BX63 microscope.

Cell invasion assays

Assays of invasion of human cell lines were performed as described (Reis et al., 2010). Briefly, cells were grown to confluent monolayers in Eagle's medium with L-glutamine (Lonza), supplemented with nonessential amino acids (Lonza), sodium pyruvate (Lonza) and 20% fetal bovine serum (FBS, Biowest) (Caco-2, ATCC HTB-37) or in DMEM with glucose (4.5 g/l) and L-glutamine (Lonza), supplemented with 10% FBS (HeLa, ATCC CCL-2 and Jeg-3, ATCC HTB-36). *Lm* was grown to an exponential phase, washed and inoculated at a multiplicity of infection of 75 for 1 h. Cells were incubated with medium supplemented with 20 µg/ml gentamycin (Lonza) for 1.30 h to eliminate extracellular bacteria, washed and finally lyzed with 0.2% Triton X100. Bacterial suspensions were serially diluted and plated on BHI-agar plates for CFU determination.

Animal infections

Animal infections were performed with 6 to 9 week-old specific pathogen-free female BALB/c mice (Charles River Laboratories) maintained at the IBMC animal facilities, in high efficiency particulate air (HEPA) filter-bearing cages under 12 h light cycles and in an *ad libitum* regiment of sterile chow and autoclaved water. Intravenous infections were performed by inoculation of 10^4 CFUs through tail vein injection as described (Cabanes et al., 2008). For oral infections mice were starved for 8-12 h before the procedure and inoculated with 2 x 10^9 CFUs (in PBS with 150 mg/ml $CaCO_3$) by gavage under light anesthesia. Mice were sacrificed by general anesthesia 72 h post-infection and the liver and spleen of each animal were aseptically removed and homogenized in PBS. Organ homogenates were serial diluted and plated in BHI-agar plates for CFU counting. 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 Alimentação e Veterinária, the Portuguese authority for animal protection, under license 015302.

Statistical analyses

Statistical analyses were performed with the software Prism 7 (GraphPad Software). Means of two groups were compared by unpaired two-tailed Student's t-test. Differences with a calculated p-value above 0.05 were considered non-significant and statistically significant differences were noted as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

lmo0443 encodes a potential LCP transcriptional regulator

We have previously reported the *in vivo* transcriptome profiling of *Lm* while infecting the mouse spleen. In that study we identified *lmo0443,* a gene predicted to encode a transcriptional regulatory protein, to be significantly more expressed during spleen infection when compared to growth in BHI medium (Camejo et al., 2009). Lmo0443 was predicted by bioinformatic and database analysis as a transcriptional regulator of the LytR family of transcription factors (Figure 1), which belongs to the LCP superfamily.

Lmo0443 is needed for virulence *in vivo*

To determine the roles of Lmo0443 in *Lm* pathogenicity we constructed a deletion mutant for the regulator-encoding gene (Δ*lmo0443*). We verified that the growth curve of Δ*lmo0443* was comparable to that of the *Lm* wild type EGD-e (WT) strain (Figure 2A), demonstrating that the in-frame deletion of *lmo0443* did not impair its growth in BHI. We then infected the human cell lines Caco-2, Jeg-3 and HeLa with *Lm* WT and Δ*lmo0443* strains and evaluated bacteria invasion by determining intracellular CFUs at 90 min post-infection. Our results show that, as compared to the WT, Δ*lmo0443* has no significant impairment on the invasion of Caco-2, Jeg-3 and HeLa cells (Figure 3A). To study the role of Lmo0443 *in vivo* we infected BALB/c mice with *Lm* WT and Δ*lmo0443* strains either orally or by intravenous inoculation and evaluated the bacterial load in spleens and livers 72 h post-infection. We observed that the mutant strain Δ*lmo0443* had an attenuated virulence phenotype in both organs and routes of infection, when compared to the WT strain (Figure 3B). The differences of the bacterial loads between the strains were higher and statistically significant after intravenous inoculation. Our data indicates that Lmo0443 is required for *Lm* virulence in the mouse model.

Lmo0443 represses the expression of *lmo0444*

Next, we intended to further characterize the role of the putative regulator Lmo0443 and determine which genes have their expression under its control. To this end, we designed an RNAseq-based whole-genome transcriptomic approach, to determine differential gene expression between *Lm* WT and the Δ*lmo0443* deletion mutant in exponential growth phase in BHI at 37°C. Our analysis revealed that the genes *lmo0444 and lmo2685* are significantly more expressed in the Δ*lmo0443* mutant strain, when compared to the WT (Figure 4A). These data suggest that the putative regulator Lmo0443 functions as a transcriptional repressor. To

validate the transcriptomic data from the RNAseq method we performed RT-qPCR on cDNA of a subset of genes from WT and Δ*lmo0443* bacteria grown to the exponential phase (Figure 4B). Data from both methods showed a good level of correlation (R^2=0.83), validating the differential levels of expression observed for *lmo0444*. However, we were not able to validate RNAseq data regarding *lmo2685* expression, which by RT-qPCR was no different from the WT. We decided to further investigate the role of *lmo0444* repression by Lmo0443 *in Lm* virulence.

Overexpression of *lmo0444* leads to growth and morphological defects in *Lm*

We hypothesized that the regulation of *lmo0444* could be the link between Lmo0443 and its role in *Lm* virulence. The gene *lmo0444* encodes a putative uncharacterized protein, is located immediately downstream of *lmo0443* and is the first of a five-gene group that constitutes a genetic element called stress survival islet (SSI-1). Since our data indicate that *lmo0444* is under repression by Lmo0443 in *Lm* WT, we constructed an *Lm* strain overexpressing *lmo0444* (++*lmo0444*) in order to mimic a lack of control by Lmo0443 and, thus, try to evaluate its role in *Lm* virulence. By monitoring the growth of the overexpression mutant ++*lmo0444* in BHI we observed that its growth curve is not comparable to that of the WT (Figure 2B). Despite reaching the population density close to the WT in the stationary phase, the lag and early exponential phases of growth of the ++*lmo0444* strain show a delay compared to the WT. To further study this growth defect, we performed a microscopic analysis *Lm* WT, Δ*lmo0443* and ++*lmo0444* cells (Figure 5A). We observed that ++*lmo0444* bacteria show abnormal morphological traits and cell division. As opposed to the WT and Δ*lmo0443* bacteria that presented the typical individualized or short chains of regular rod-shaped *Lm* cells, in the ++*lmo0444* mutant a high portion of the population presented an atypical and irregular shape, more chained cells, more clusters of aggregated cells and cells were observed to be less motile. In addition to this, we grew cultures of *Lm* WT, Δ*lmo0443* and ++*lmo0444* strains in BHI to the stationary phase and let the culture stand with no agitation for two days. We observed that the growth medium of the mutant ++*lmo0444* culture cleared more quickly and the bacteria were deposited in the bottom of the culture tubes faster (Figure 5B). Altogether, our results indicate that whereas Lmo0443 might regulate *Lm* virulence through the repression of *lmo0444*, a higher expression of *lmo0444* induces severe defects in growth, shape and motility which are not observed in the deletion mutant Δ*lmo0443.*

Discussion

The discovery and characterization of novel virulence regulators is crucial to better understand how pathogenic bacteria modulate the expression of their genomes, fine tune the virulome in order to adapt to the host environment and establish infection (Freitag et al., 2009). Here we identify *lmo0443* as a putative virulence transcription regulator encoding gene of *Lm*. We found that *lmo0443* was upregulated while infecting mice spleen, as compared to when multiplying in BHI (Camejo et al., 2009). This prompted us to evaluate if this putative transcription factor had a role in *Lm* virulence. Indeed, we demonstrated that Lmo0443 is necessary for full virulence in the mouse model. Interestingly *lmo0443* has been previously reported to be less expressed in more virulent *Lm* strains while being overexpressed in less virulent strains (Dumas et al., 2008). Our *in vitro* and *in vivo* results obtained with the Δ*lmo0443* mutant are not in concordance with what was reported and rather seem to indicate that less or no expression of *lmo0443* develops a less virulent phenotype. Lmo0443, a protein found in the exoproteome of *Lm*, is a putative LCP regulator, a family of regulatory attenuators with roles across Gram-positive bacteria including virulence regulation (Desvaux et al., 2010; Hübscher et al., 2008). Interestingly, according to our transcriptomics data, Lmo0443 also appears to act a transcriptional attenuator, by repressing the expression of *lmo0444*. This gene belongs to the SSI-1 islet, a genomic element of *Lm* which has been associated with tolerance to different stresses (i.e. low pH, high salt and bile) (Begley et al., 2005; Cotter et al., 2005; Ryan et al., 2010). To our knowledge, of the five SSI-1 genes (*lmo0444-lmo0448*) *lmo0444* seems to remain the only one lacking any proper characterization. Whereas *lmo0446* (*pva*)*, lmo0447* (*gadD1*) *and lmo0448* (*gadT1*) have been linked to stress tolerance (Begley et al., 2005; Cotter et al., 2005; Ryan et al., 2010), *lmo0445* has been described has a positive transcription regulator of the SSI-1 genes (Ryan et al., 2010). Although the SSI-1 lacks any of the traditional mobility factors (i.e. integrases, transposases, insertion sequence elements), given that Lmo0444 protein sequence shows some homology to a phage infection protein (Pip), it has been suggested that the role of Lmo0444 could be related to how the islet is acquired in the genome (Ryan et al., 2010). Our results, however, suggest for the first time that Lmo0444 could have other specific roles in the cell. In fact, we showed that higher levels of *lmo0444* have dramatic effects on cell morphology and division, which could be related to a role of Lmo0444 at the level of the cell envelope. Interestingly, it appears that increasing evidence has recently risen linking LytR and other LCP to cell wall maintenance functions, either by transcription regulation of other factors or by directly catalyzing enzymatic reactions at the cell wall (Baumgart et al., 2016; De et al., 2017; Eberhardt et al., 2012; Gale et al., 2017; Minami et al., 2012; Wang et al., 2015). Indeed, LCP proteins are also predicted to have a transmembrane domain and Lmo0443 is even a secreted protein, which shows association to

the cell wall. In addition to this, *lmo0443* has been shown to be one of the CesRK two-component system induced genes to be upregulated in response to the presence of antibiotics that target cell wall biosynthesis like β-lactams (ampicillin and cefuroxime) and vancomycin (Gottschalk et al., 2008; Kallipolitis et al., 2003; Knudsen et al., 2012; Nielsen et al., 2012). For this reason, deletion mutants for many of these LCP proteins develop phenotypical defects in growth, cell division, cell morphology and motility. Curiously, these exact kinds of defects were observed in *Lm* overexpressing *lmo0444* but not in deletion mutant for Lmo0443. Considering the mutant ++*lmo0444* cells altered cell shape and impaired cell division, we hypothesize that increased Lmo0444 production probably causes a dramatic change at the level of the cell wall, rather than the impairment of a metabolic pathway. This could indicate a novel cell wall-related role for Lmo0444 and also suggests that Lmo0443 plays a cell wall maintenance-related role by transcription regulation of *lmo0444*. Unfortunately, the severe growth and morphology defects of the ++*lmo0444* mutant compromised further phenotypical characterization of the role of Lmo0444 in *Lm* virulence and deeper characterization of the Lmo0443 virulence regulatory network. Further studies would therefore be needed to characterize these interactions.

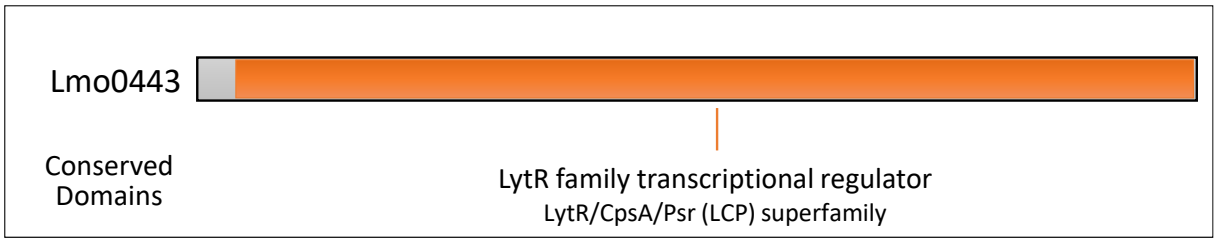

Figure 1. *lmo0443* encodes a putative LytR transcription factor. Bioinformatic analysis predicts the locus *lmo0443* of *Lm* to encode a transcription factor of the LytR family of the LCP superfamily of proteins.

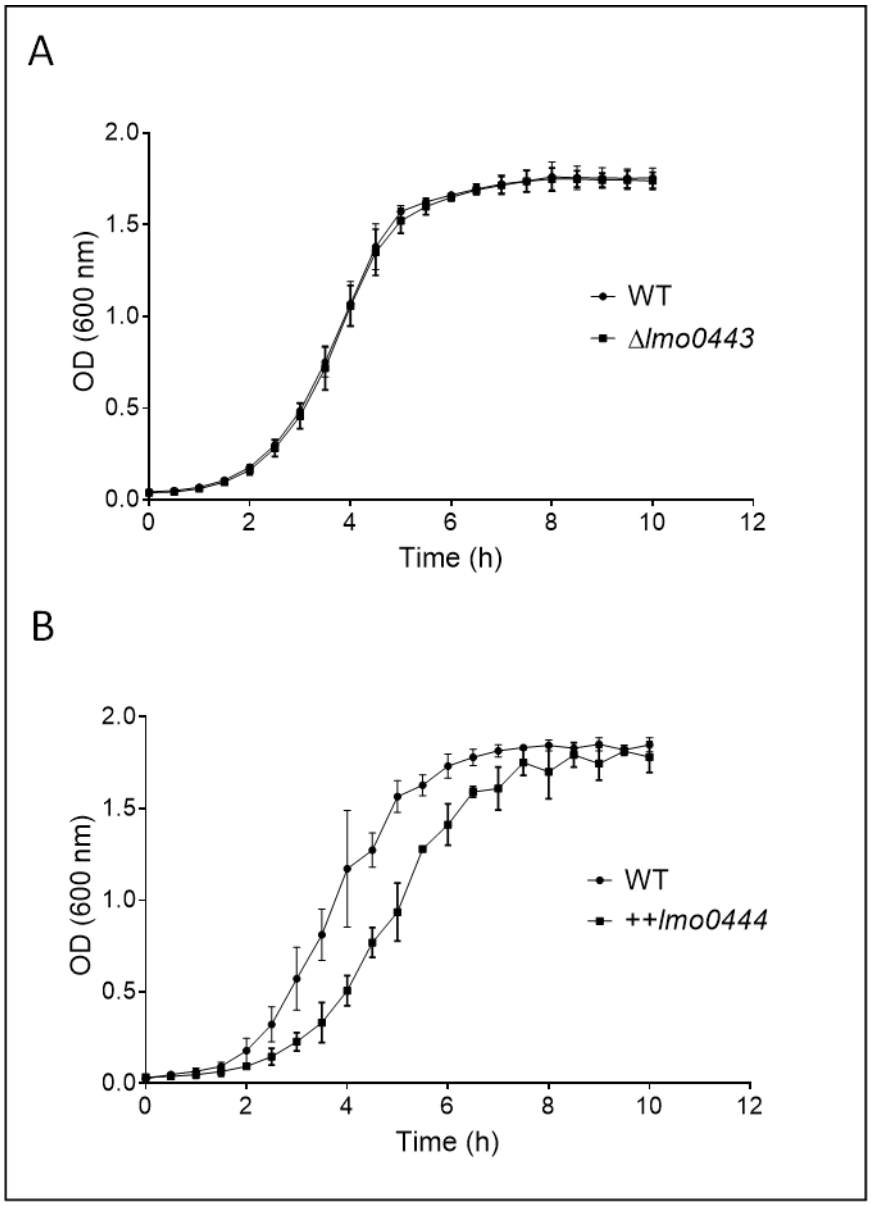

Figure 2. Growth profile of *Lm* strains in BHI medium. Overnight cultures of *Lm* WT and **(A)** Δ*lmo0443* or **(B)** ++*lmo0444* were diluted 100-fold in BHI medium and incubated at 37°C with constant agitation. Measurement of optical density (OD_{600nm}) was performed every 30 min. Results are mean ± SD of three independent experiments.

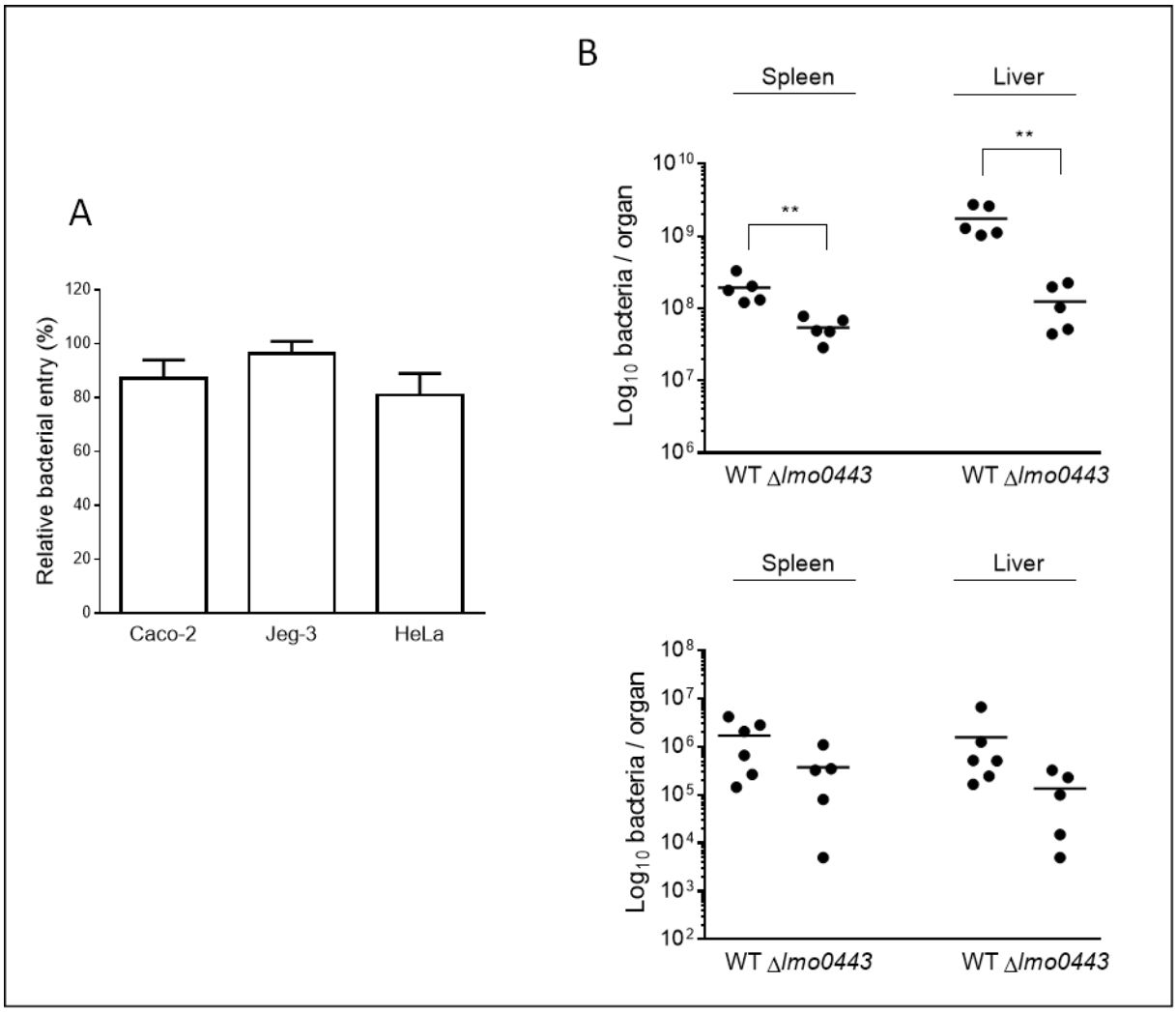

Figure 3. Lmo0443 is required for virulence. **(A)** Invasion of *Lm* WT and Δ*lmo0443* into Caco-2, Jeg-3 and HeLa cell monolayers, represented as intracellular CFU counts relative to WT (fixed at 100%). Results are mean ± SD of three independent experiments. **(B)** CFU counts in spleens and livers of female BALB/c mice 72 h after (top) intravenous infection with 10^4 CFU of WT or Δ*lmo0443* or (bottom) oral infection with 10^9 CFU of WT or Δ*lmo0443*. Each dot of the plot corresponds to one animal, mean values are represented by a horizontal bar. **, $p \leq 0.01$.

A

Gene	Fold change†
lmo0444	+ 5,19
lmo2685	+ 4,05

† Fold change values refer to expression of genes significantly regulated in *Lm* Δ*lmo0443* as shown by *in vitro* transcriptome vs. WT.

B

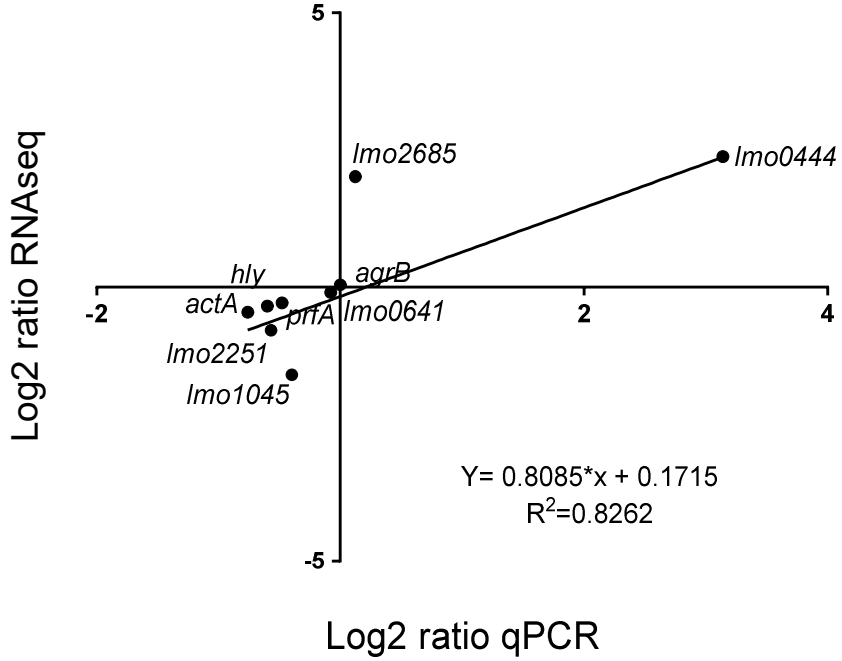

Figure 4 Genes differently regulated in Δ*lmo0443 in vitro.* **(A)** Fold change of the expression of genes regulated by Lmo0443 as shown by RNAseq. **(B)** Validation of RNAseq transcriptome data by RT-qPCR. Data represented as Log2 of fold change between *in vitro* gene expression of *Lm* WT and Δ*lmo0443* in exponential growth in BHI at 37°C as measured by RNAseq and RT-qPCR (*lmo2685* that was not validated by RT-qPCR, not accounted for linear regression).

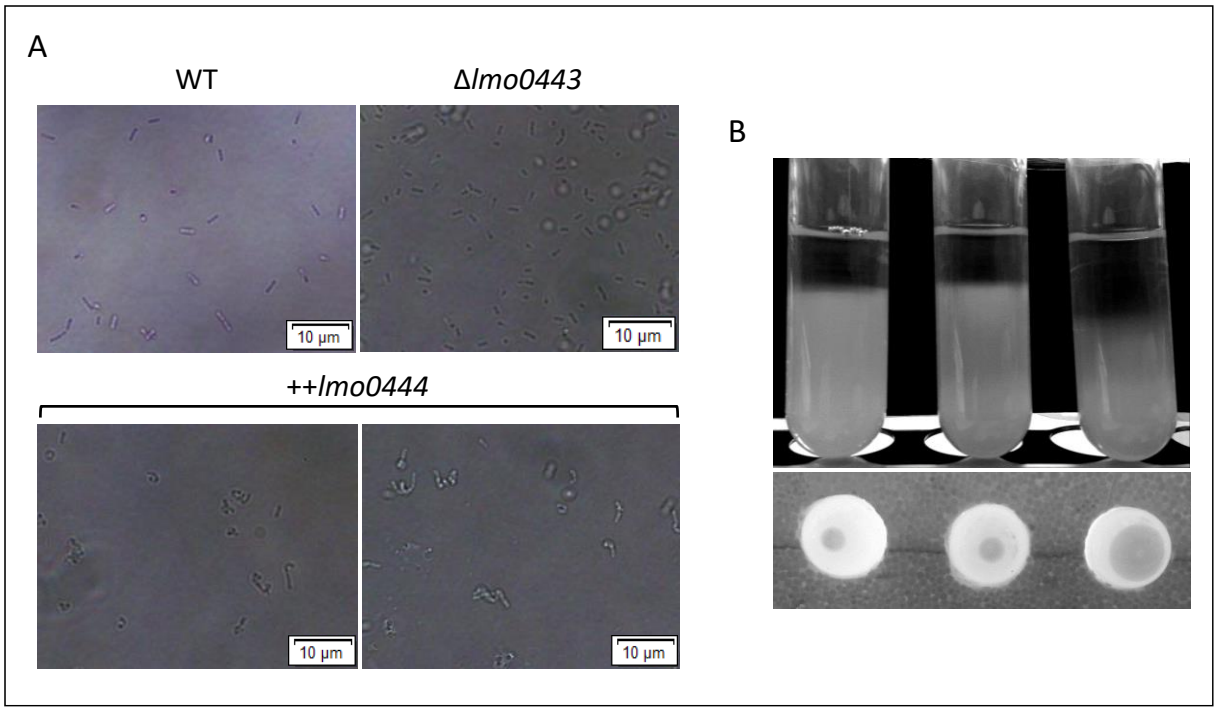

Figure 5. Overexpression of *lmo0444* causes morphological defects in *Lm*. **(A)** Bright-field microscopy imaging of *Lm* WT, Δ*lmo0443* or ++*lmo0444* cells grown in BHI. **(B)** Lateral and bottom view of stationary phase cultures of (from left to right) *Lm* WT, Δ*lmo0443* or ++*lmo0444* in BHI, after two days without agitation.

Table 1 - Plasmids and bacterial strains

Plasmid/Strain	Description	Source
Plasmids		
pMAD	Gram-negative/Gram-positive shuttle vector; thermosensitive replication; Amp^r Ery^r	Arnaud et al., 2004
pMAD(Δ*lmo0443*)	*pMAD* with 5'- and 3'-flanking regions of *lmo0443* locus; Amp^r Ery^r	This study
pIMK-2	*L. monocytogenes* phage-derived site-specific integration overexpression vector; Kan^r	Monk et al., 2008
pIMK-2(*lmo0444*)	pIMK-2 with the *lmo0444* locus; Kan^r	This study
E. coli		
DH5α	Cloning host strain; F^- Φ80*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Life Technologies
Listeria monocytogenes		
EGD-e	Wild type; serotype 1/2a	Glaser et al., 2001
EGD-e Δ*lmo0443*	EGD-e *lmo0443* deletion mutant	This study
EGD-e ++*lmo0444*	EGD-e harboring pIMK-2(*lmo0444*); Kan^r	This study

Table 2 - Primers

Name	Sequence (5' - 3')
*Plasmid/mutant construction**	
mouR- A	CGCGTCGACCGGCTTGGTACTTGTCTTCC
mouR- B	CGACGCGTCATATTTCACACCTCTTTC
mouR- C	GCACGCGTTAAAGCAGAACACGGAAATC
mouR- D	GACCATGGTCCGCTGCTTGTACAATGCCA
mouR- F	CGGTCCAGAACTTGTACGCC
mouR- R	CGAAGTACTGCTCCCGCTCCATC
pIMK-2-*lmo0444*-F	GTTTCCATGGTTAATATGGTCAAAAATGAGTGG
pIMK-2-*lmo0444*-R	CGTGCGTCGACTTAATCTTCTACTGCTTGTAATTC
PL95	ACATAATCAGTCCAAAGTAGATGC
PL102	TATCAGACCTAACCCAAACCTTCC
RT-qPCR	
qPCR-*16S*-F	GCGTAGATATGTGGAGGAAC
qPCR-*16S*-R	CAGGCGGAGTGCTTAATG
qPCR-*actA*-F	CGAGCCTACCAGTAATCC
qPCR-*actA*-R	CTGATTCGCTTTCCTCTACC
qPCR-*agrB*-F	TCAGAAAGAATGGCGGATG
qPCR-*agrB*-R	CCTGTTACTAAGGCGATACC
qPCR-*hly*-F	TCGTCCATCTATTTGCCAGG
qPCR-*hly*-R	TTACCGTTCTCCACCATTCC
qPCR-*lmo0444*-F	AGCCATTACAAGCGGAAT
qPCR-*lmo0444*-R	CGAGCCATAGTAGTCCAAT
qPCR-*lmo0641*-F	CAATTCAAGCCGAACTAGG
qPCR-*lmo0641*-R	CACACTGCCATAAGTAACC
qPCR-*lmo1045*-F	GCTGAAAAGACACATAAAACCG
qPCR-*lmo1045*-R	CACTACTAATAACTTCTCGGACCT
qPCR-*lmo2251*-F	GAGAAGTGGTCGTAGTTATCG
qPCR-*lmo2251*-R	GGTTTGTTTCGCCATTAGG
qPCR-*lmo2685*-F	CTATGTTAGCCATTGATTCCG
qPCR-*lmo2685*-R	AGTAGTGCTTGTTCTGCTT
qPCR-*prfA*-F	CGAGTATTAGCGAGAACG
qPCR-*prfA*-R	GATAACGTATGCGGTAGC

* Restriction sites are underlined

PART III

Listeria monocytogenes encodes a functional ESX-1 secretion system whose expression is detrimental to *in vivo* infection

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RESEARCH PAPER

Listeria monocytogenes encodes a functional ESX-1 secretion system whose expression is detrimental to *in vivo* infection

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ABSTRACT

Bacterial pathogenicity deeply depends on the ability to secrete virulence factors that bind specific targets on host cells and manipulate host responses. The Gram-positive bacterium *Listeria monocytogenes* is a human foodborne pathogen that remains a serious public health concern. To transport proteins across its cell envelope, this facultative intracellular pathogen engages a set of specialized secretion systems. Here we show that *L. monocytogenes* EGDe uses a specialized secretion system, named ESX-1, to secrete EsxA, a homolog of the virulence determinants ESAT-6 and EsxA of *Mycobacterium tuberculosis* and *Staphylococcus aureus*, respectively. Our data show that the *L. monocytogenes* ESX-1 secretion system and its substrates are dispensable for bacterial invasion and intracellular multiplication in eukaryotic cell lines. Surprisingly, we found that the EssC-dependent secretion of EsxA has a detrimental effect on *L. monocytogenes in vivo* infection.

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ESAT-6; ESX-1 secretion system; EsxA; *Listeria monocytogenes*; Type VII secretion system

Introduction

The capacity to secrete proteins is crucial for the pathogenesis of many bacteria. In Gram-positive bacteria, proteins are delivered by highly specialized secretion systems across cell envelope to reach specific targets.[1] A portion of these proteins, including virulence factors, are often secreted by Sec-independent systems. ESX-1, also called WXG100, is a Sec-independent secretion system first described in *Mycobacterium tuberculosis*.[2-4] This system allows the secretion of ≈100 amino-acid-long proteins that lack the classical signal peptide but contain a Trp-X-Gly motif (WXG100 proteins). *M. tuberculosis* ESAT-6 and CFP-10 are prototypes of WXG100 proteins, both encoded by the region of difference 1 (RD1), involved in virulence and described as highly immunogenic proteins.[5] Deletion of the *esx-1* locus abrogates ESX-1-dependent secretion and strongly attenuates the virulence of *M. tuberculosis*.[6] Apart from mycobacterial species, ESX-1 systems are also found in Firmicutes, among which the *S. aureus* ESX-1 is one of the best-characterized.[7,8] It comprises genes encoding the canonical ESX-1 substrates (EsxA, EsxB), a membrane-anchored FtsK/SpoIIIE-like ATPase (EssC) essential for the secretion machinery, genes coding for membrane-embedded proteins (EssA, EssB, EssD) required for secretion of ESX-1 substrates, as well as staphylococci-specific ESX-1 substrates (EsxC, EsxD), and modulators of ESX-1 activity (EsaA, EsaB).[7-10] *S. aureus* EsxA and EsxB share features with the *M. tuberculosis* ESAT-6 and CFP-10, including the presence of a WXG motif and the co-dependent secretion. However, unlike ESAT-6 and CFP-10, EsxA and EsxB do not interact. EsxA dimerizes with itself or associates with EsxC, while EsxB interacts with EsxD.[8] Disruption of key components of the *S. aureus* ESX-1 secretion machinery (EssC) or deletion of *esxA* and *esxB* causes a significant reduction in the ability of *S. aureus* to establish kidney or liver abscesses.[7,9,11] Moreover, the *S. aureus* ESX-1 secretion system is required for nasal colonization and virulence in a murine lung pneumonia model.[12] EsxA was also shown to interfere with host cell apoptotic pathways, affecting bacterial survival and mediating *S. aureus* release from

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host cells.[13] Other ESX-1 substrates, such as EsaC, although dispensable for the establishment of acute infections, are required for the formation of persistent infection.[11] Functional ESX-1 secretion systems were also characterized in *Bacillus anthracis, Bacillus subtilis, Actinobacterium* and *Streptomyces coelicolor*.[14-17]

Listeria monocytogenes (*Lm*) is a ubiquitous Gram-positive bacterium responsible for listeriosis, a severe opportunistic foodborne disease occurring mainly in immunocompromised individuals, newborn, elderly and pregnant women. Listeriosis is the most frequent cause of hospitalization and death due to the consumption of contaminated food in Europe, and involves high illness costs and quality life losses.[18] Clinical features of listeriosis includes septicemia, meningitis, meningoencephalitis and abortions. This facultative intracellular pathogen has evolved multiple strategies to survive inside phagocytic cells, invade non-phagocytic cells and spread from cell to cell.[19] Each step of its cell infection cycle depends on specific virulence determinants that play specific roles, most of them being surface or secreted proteins.[20,21] Genes encoding a potential ESX-1 secretion system were identified in *Lm*[22] and the *Lm* EsxA homolog was previously shown dispensable for *Lm* mouse infection.[23] However, although ESX-1 was postulated to represent a broad Gram-positive secretion system,[1] the functionality of this apparatus and its role in infection were never previously investigated in *Listeria*.

Results

Lm *encodes a putative ESX-1 secretion system*

In agreement with the model proposed for the ESX-1 secretory apparatus[1,22] and following bioinformatic analyses (BLAST, TopPred2, ProDom) we determined that the *Lm* ESX-1 locus contains genes coding for: the 2 canonical WXG100 substrate paralogs EsxA (Lmo0056) and EsxB (Lmo0063), the integral membrane FtsK/SpoIIIE-type ATPase EssC (Lmo0061); EsaA (Lmo0057), a polytopic membrane protein with 5 predicted transmembrane helices; EssA (Lmo0058) and EssB (Lmo0060), 2 predicted membrane proteins with respectively one and 2 transmembrane domains; and EsaB (Lmo0059) and Lmo0062, 2 putative cytoplasmic proteins (Fig. 1A). Membrane topology or soluble character of proteins encoded by the *Lm* ESX-1 locus was predicted and compared to *M. tuberculosis* and *S. aureus* ESX-1 secretion systems (Fig. 1B). This revealed large similarities with the *S. aureus* ESX-1 systems. However, the 2 ESX-1 substrates (EsxC and EsxD) and the EssD transmembrane protein described in *S. aureus* are absent in *Lm*. Analysis of complete genome sequences available for different *Listeria* species revealed the high level of conservation of the ESX-1 locus within the *Listeria* genus (Fig. 1A). Of note, *esxB* and *lmo0062* are absent from 3 non-pathogenic species (*L. welshimeri, L. seeligeri* and *L. grayi*).

Lm *ESX-1 secretion system is functional although weakly expressed*

To evaluate the expression of the *Lm esx-1* locus, we analyzed by RT-PCR the transcription of genes encoding the 2 major ESX-1 effectors (*esxA* and *esxB*), and 2 integral membrane proteins essential for the secretion machinery (*essB* and *essC*).[7-10] RNAs extracted from bacteria in exponential growth phase in BHI at 37°C were processed for analysis and results showed that all the genes selected are transcribed in these conditions (Fig. 2A). However, *esxB, essC* and *essB* appeared to be weakly expressed as compared to *esxA* and control genes (*inlA, actA, iap*) encoding known *Lm* virulence factors. We also observed that the expression of *esxA* appeared to decrease upon entry into stationary growth phase, which was confirmed by qRT-PCR (Fig. 2B). We also attempted to assess by qRT-PCR the expression of *esxB* and *essC* during growth in exponential or stationary phases. However, transcript levels appeared insufficient to obtain quantifiable amplification, in particular during the stationary growth phase, thus underlining the weak level of expression of these genes. *esxA* was previously shown to be negatively regulated by SigB in *S. aureus*.[24] SigB being the major regulator of the stationary growth phase, we assessed if the decreased expression of *esxA* upon entry into this phase would be due to SigB regulation. We analyzed by qRT-PCR the expression of *esxA* in a *sigB* deletion mutant as compared to the WT strain during growth in exponential and stationary phases, and showed that in both growth phases *esxA* expression appeared independent of the presence of *sigB* (Fig. 2C). *bsh,* which encodes a bile salt hydrolase, was used as a SigB-dependent control gene.[25]

To analyze whether the putative *Lm* ESX-1 system was functional, we constructed a deletion mutant strain for *essC* (Δ*essC*), which encodes a structural protein essential for *S. aureus* ESX-1 functionality,[7] as well as the corresponding complemented strain (Δ*essC*+*essC*). Both strains were confirmed by PCR and sequencing, and their growth rate in BHI at 37°C was comparable to that of the WT (Fig. S1A-B). To test the functionality of the ESX-1 secretion system in *Lm*, we expressed a myc-tagged EsxA protein (EsxA-myc) in WT bacteria and analyzed its secretion during bacterial growth. Western blot of bacterial culture supernatants showed a band of 11 kDa, the expected size for EsxA-myc, with a higher intensity in the exponential growth phase (Fig. 2D left panel). These results indicate that *Lm* EsxA was secreted in these

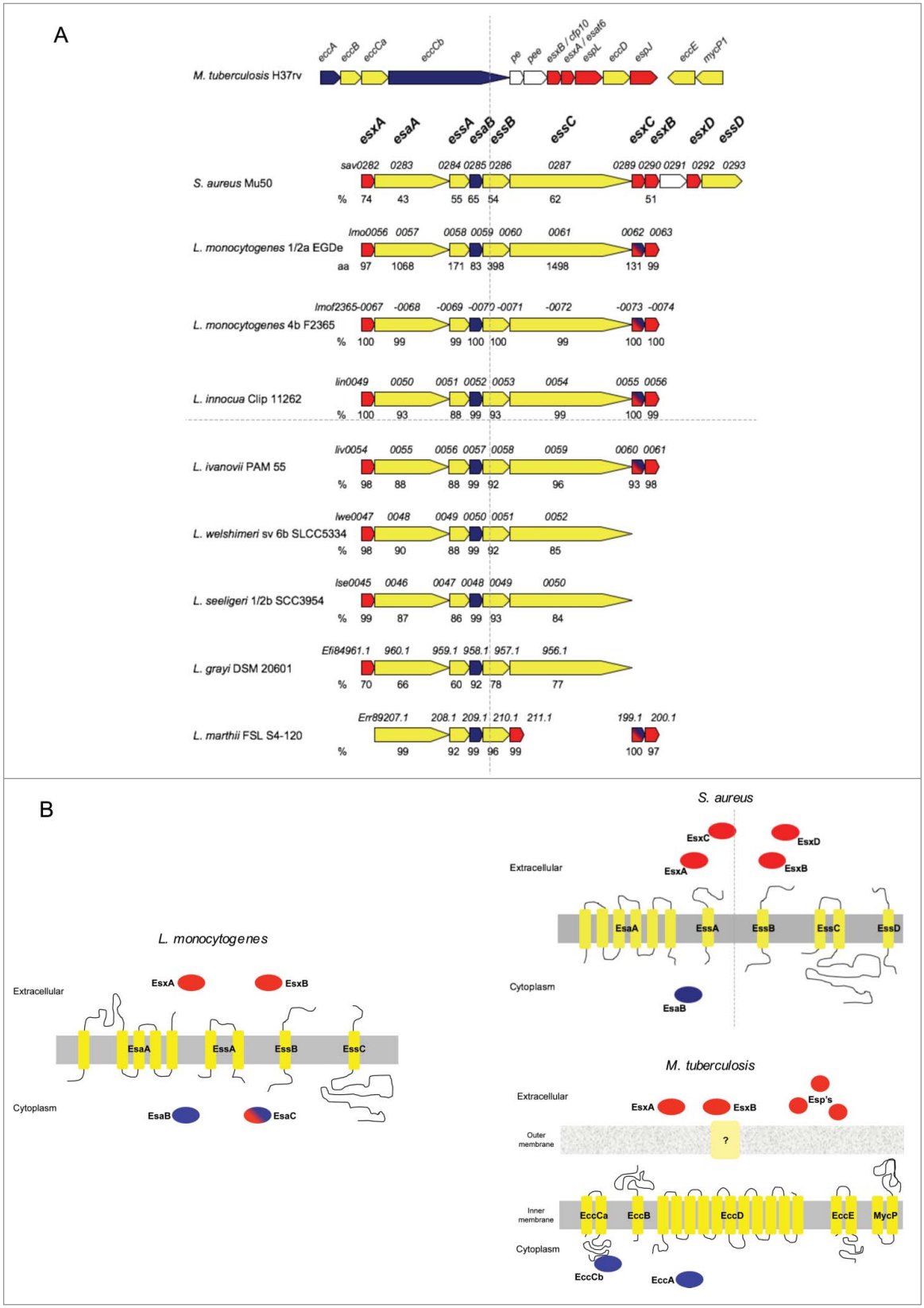

Figure 1. The ESX-1 locus. (A) Comparison of ESX-1 loci of *M. tuberculosis, S. aureus, L. monocytogenes* EGDe, and other *Listeria* species as indicated. Protein homology percentages relative to *L. monocytogenes* EGDe are indicated under each corresponding encoding gene. (B) Schematic representation showing membrane topology or soluble character of proteins encoded by the *L. monocytogenes, S. aureus* and *M. tuberculosis* ESX-1 locus. (A and B) Genes and proteins are colored following the same code: red corresponds to WXG100 encoding genes or proteins predicted to be secreted to the extracellular medium; blue indicates genes predicted as encoding soluble cytoplasmic proteins; yellow is related to genes or proteins predicted as transmembrane proteins.

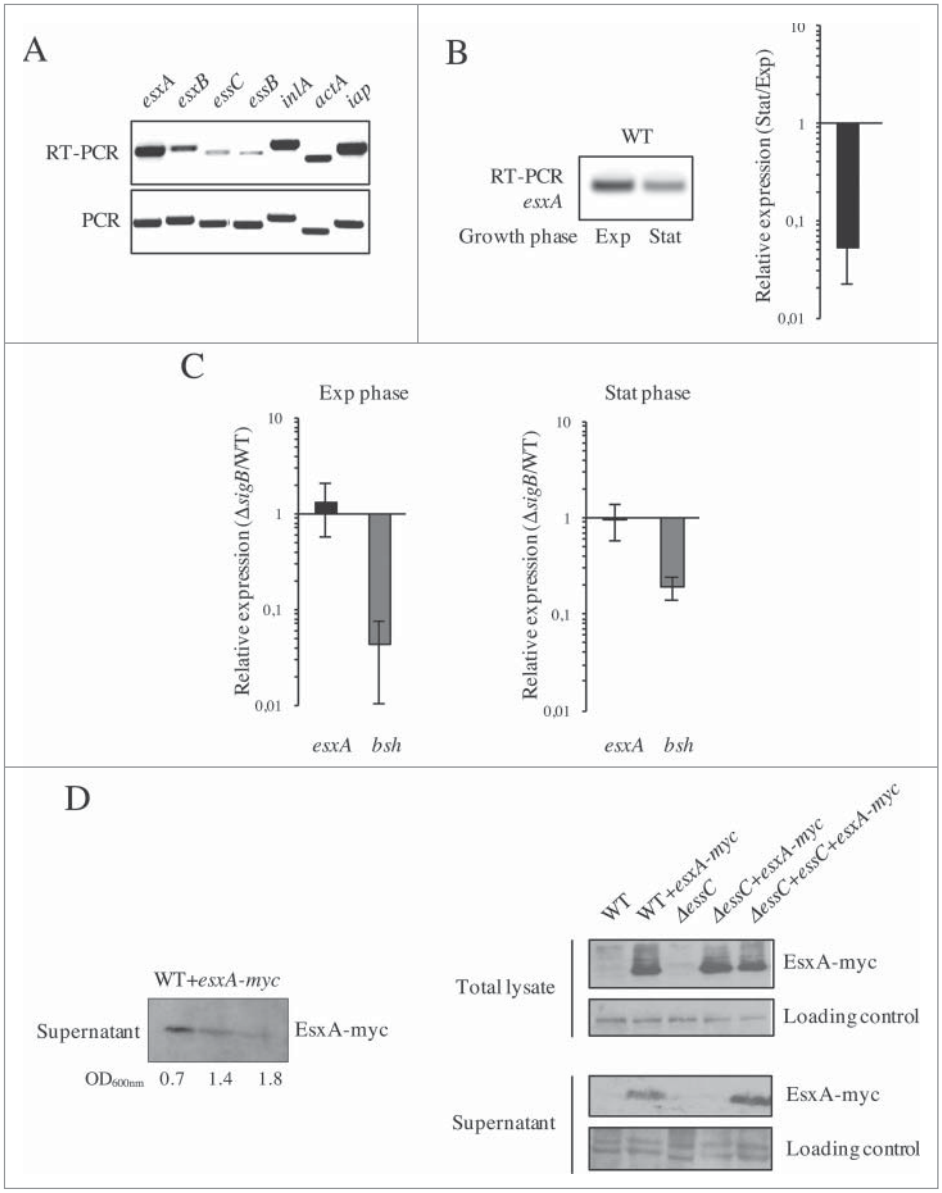

Figure 2. *Lm* ESX-1 system is weakly expressed but functional. (A) Expression of ESX-1 genes in standard growth conditions. The expression of *esxA, esxB, essC* and *essB* was analyzed by RT-PCR on total RNAs extracted from logarithmic cultures grown in BHI at 37°C. *inlA, actA* and *iap* were used as control genes. (B) Expression of *esxA* at exponential (Exp) and stationary (Stat) phase of growth measured by RT-PCR (left panel) and qRT-PCR (right panel). Expression value in stationary phase is expressed relative to the value obtained in exponential growth phase. (C) SigB-independent expression of *esxA*. qRT-PCRs performed on total RNAs extracted from WT and Δ*sigB* strains at the exponential (left panel) and stationary (right panel) phase of growth in BHI at 37°C. *bsh was* used as control gene whose expression is SigB-dependent. Gene expression levels in the Δ*sigB* mutant were normalized to those in the WT. (B and C) Values are mean ± SD (n = 3). (D) Secretion of EsxA is dependent on EssC. Detection of myc-tagged EsxA protein (EsxA-myc) in supernatants of *Lm* EGDe+*esxA-myc* (WT+*esxA-myc*) at different stages of growth (OD_{600nm} = 0.7, 1.4 and 1.8) (Left panel), and in total bacterial lysates and supernatants from WT, WT+*esxA-myc*, Δ*essC*, Δ*essC*+*esxA-myc* and Δ*essC*+*essC*+*esxA-myc* strains in exponential growth phase (Right panel).

conditions. We then expressed EsxA-myc in the Δ*essC* and Δ*essC*+*essC* strains and performed the same western blot analysis on proteins from the WT, WT+*esxA-myc*, Δ*essC*, Δ*essC*+*esxA-myc* and Δ*essC*+*essC*+*esxA-myc* total bacterial lysates and culture supernatants in exponential growth phase. In the WT+*esxA-myc* strain, EsxA-myc was detected both in the total lysates and culture supernatants, indicating that the protein is produced and secreted (Fig. 2D right panel). In the Δ*essC*+*esxA-myc* strain, EsxA-myc was absent from the supernatant and retained in total lysates, demonstrating that EsxA is secreted in an EssC-dependent manner. This was confirmed by the complementation of the Δ*essC*+*esxA-myc* mutant that restored the secretion of EsxA-myc

(Δ*essC*+*essC*+*esxA-myc* line). Altogether, these results show that *Lm* expresses a functional ESX-1 secretion system, albeit at low levels, and that EsxA secretion requires the putative membrane ATPase EssC.

Lm *ESX-1 is dispensable for host cell invasion and intracellular multiplication*

To investigate the role of the ESX-1 system in *Lm* cell invasion and intracellular multiplication, we constructed deletion mutants for *esxA*, that we showed to encode a substrate of *Lm* ESX-1, and *esxB* that encodes another putative ESX-1 substrate.[7] Mutants were confirmed by PCR and sequencing, and their growth rates observed in BHI at 37°C were comparable to that of the WT (Fig. S1A-B).

The WT, Δ*esxA,* Δ*esxB* and Δ*essC* strains were tested for their capacity to invade epithelial cell lines in which *Lm* entry is mainly mediated by internalin A (InlA) (Caco-2) or InlB (Vero). No significant difference in invasion was observed between mutant and WT bacteria in both cell lines (Fig. 3A), suggesting that the ESX-1 system is not required for *Lm* invasion of epithelial cells.

To analyze the role of ESX-1 in *Lm* intracellular multiplication, the behavior of the WT, Δ*esxA,* Δ*esxB* and Δ*essC* strains was studied after internalization in J774 murine macrophage-like cells. All strains grew with similar multiplication rates after uptake (Fig. 3B), indicating that none of these genes is required for *Lm* intracellular replication in macrophage-like cells. Altogether these results indicate that the ESX-1 secretion system is dispensable for *Lm* cell invasion and intracellular multiplication.

ESX-1 activity impairs Lm *infection*

To analyze the involvement of the ESX-1 system in *Lm* infection *in vivo*, we monitored the number of bacteria in the liver and spleen of mice infected intravenously with Δ*esxA,* Δ*esxB*, Δ*essC* or WT bacteria. Unexpectedly, 72h post-infection, all the mutant strains showed a slight increase in bacterial counts in both organs as compared to WT, which was statistically significant for Δ*esxB* (Fig. 4A), implying that ESX-1 activity might have a negative impact on *Lm* infection.

To investigate the potential role of ESX-1 in the gastrointestinal phase of the infectious process, we performed oral inoculation of mice with the WT, Δ*esxA,* Δ*esxB* and Δ*essC* strains. Three days post-inoculation, mutant strains appeared again to be slightly more virulent than the WT. This increased infection was statistically significant for Δ*esxA* in mouse livers (Fig. 4B).

These data indicate that neither the ESX-1 apparatus nor its substrates are crucial for *Lm* infection in the mouse model. Conversely, they suggest that the expression/function of this secretion system causes an adverse effect in *Lm* pathogenicity.

Overexpression of esxA *in the context of a functional ESX-1 system is detrimental to* Lm *infection*

To further investigate if the ESX-1 function could have a negative effect on *Lm* infection, we performed intravenous infection of mice with *Lm* overexpressing *esxA* (+*esxA*), together with the WT and Δ*esxA* strains. The *esxA* overexpression in the +*esxA* strain was first confirmed by qRT-PCR (Fig. S2A). Growth rate of the +*esxA* strain in BHI or minimal medium at 37°C, as well as its cell adhesion and infection capacity were comparable to that of the WT strain (Fig. S2B-C), indicating that *esxA* overexpression has no significant impact on *Lm* growth and cellular infectious properties. Three days post infection the Δ*esxA* mutant appeared slightly more virulent as compared to the WT, as already observed (Fig. 4A), whereas +*esxA* bacteria showed a significant number decrease in both mouse organs (Fig. 5A). Inversely, *esxA* overexpression had no effect on the phenotype of a Δ*essC* mutant that, similarly to the Δ*esxA* mutant, also appeared to colonize more efficiently mouse organs than the WT strain (Fig. 5B). These results demonstrate that the detrimental effect of ESX-1 on *Lm* pathogenicity is due to EsxA secretion and depends on a functional ESX-1 machinery.

The production of IFN-γ and TNF-α by immune cells promotes bacterial clearance and is critical in controlling primary *L. monocytogenes* infections.[26] To investigate if the adverse effect of *esxA* overexpression on *Lm* infection is related with higher levels of host IFN-γ and/or TNF-α, we analyzed by qRT-PCR levels of IFN-γ and TNF-α transcripts in the liver of WT or +*esxA*-infected mice. No significant difference was observed regarding expression levels of IFN-γ and TNF-α (Fig. 5C). In addition, to discard any role of IFN-γ in the increased resistance of mice to *esxA* overexpressing *Lm*, WT and IFN-γ knockout mice (IFN-$\gamma^{-/-}$) were intravenously infected with WT or +*esxA* bacteria. Three days post-infection, bacterial loads were overall higher in the organs of IFN-$\gamma^{-/-}$ than in WT mice (Fig. 5D). These data indicated that IFN-γ deficient mice are more susceptible to *Lm* infection and confirmed the role of IFN-γ in the immune response against *Lm*.[27] In line with data observed in BALB/c mice (Fig. 5A), the +*esxA* strain showed significant infection attenuation in both organs of WT mice as compared to *Lm* WT bacteria (Fig. 5D). However, this infection defect was similar in IFN-γ deficient animals,

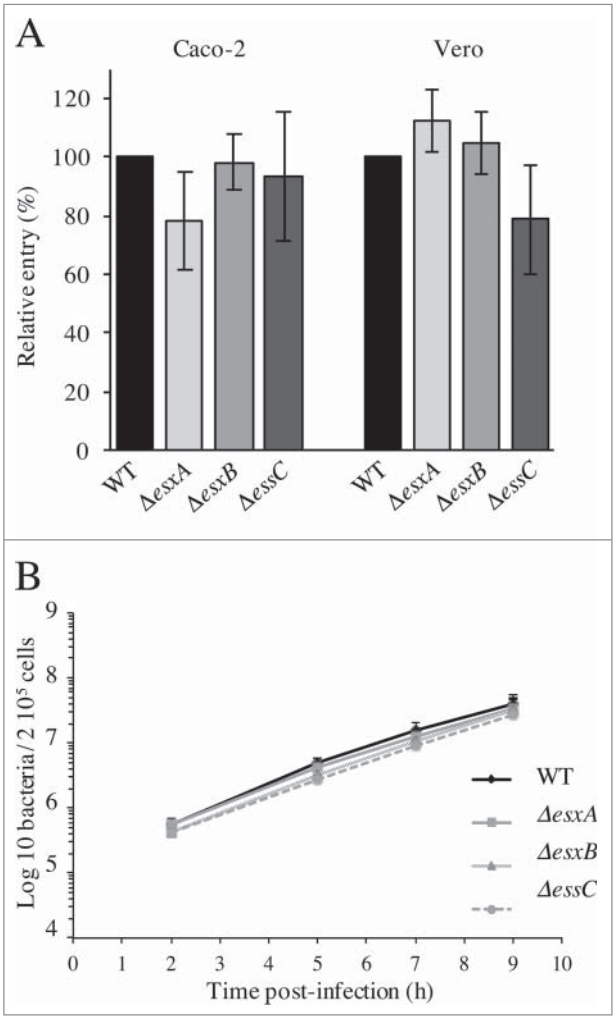

Figure 3. The *Lm* ESX-1 secretion system is dispensable for epithelial cell invasion and intracellular multiplication in macrophages. (A) Entry of the WT, Δ*esxA*, Δ*esxB* and Δ*essC* into Caco-2 and Vero cell lines. Values are expressed relative to WT values arbitrarily fixed to 100%. (B) Intracellular replication behavior of the WT, Δ*esxA*, Δ*esxB* and Δ*essC* strains in J774 cells. Values are mean ± SD (n = 3).

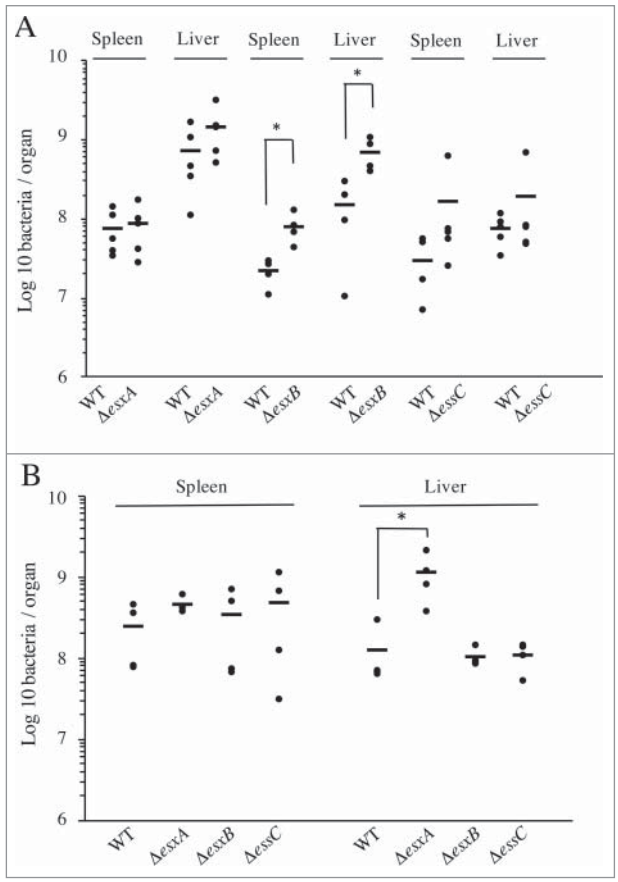

Figure 4. *Lm* ESX-1 secretion system is detrimental for *in vivo* infection. Bacterial counts for the WT, Δ*esxA*, Δ*esxB* and Δ*essC* strains, in spleens and livers of BALB/c mice (n = 5), 72h after (A) intravenous infection with 10^4 bacteria or (B) oral infection with 10^9 bacteria. Data are presented as scatter plots, which each animal represented by a dot and the mean is indicated by a horizontal line. *, $p \leq 0.05$.

suggesting that IFN-γ is not involved in the increased resistance of mice to *Lm* overexpressing *esxA*.

Discussion

The analysis of the bacterial ability to secrete proteins to or beyond their surface is crucial in the understanding of bacterial pathogenesis. In *M. tuberculosis* and *S. aureus*, ESX-1 and its substrates were shown to play an important role in virulence.[5,7] ESX-1 appears to be very conserved in *Listeria*, in particular among pathogenic species, and only partially present in the majority of non-pathogenic *Listeria* species.

We showed that the ESX-1 locus of *L. monocytogenes* EGDe is expressed in standard growth conditions, with *esxA* appearing more expressed than *esxB*, *essB* and *essC*. These results are in agreement with previous transcriptional analyses[28] and are consistent with the presence of a transcription terminator between *esxA* and *esaA*. This could suggest a different transcriptional regulation between *esxA* and the other ESX-1 genes. Regarding the expression of ESX-1 genes in different conditions, no change was observed when bacteria were grown at 37°C, 25°C or 7°C,[29,30] nor in presence of 6% NaCl,[29] nor when grown at pH 5,[31] nor when grown in culture media supplemented with glucose, cellobiose or glycerol.[32,33] In addition, as compared to bacteria grown in BHI at 37°C, the expression of the ESX-1 locus appears also unchanged in *Listeria* recovered from infected murine macrophages,[34,35] or from mouse intestinal lumen[36] and spleens.[37] Interestingly, the entire ESX-1 locus was shown as up-regulated after incubation of *Lm* in human blood,[36]

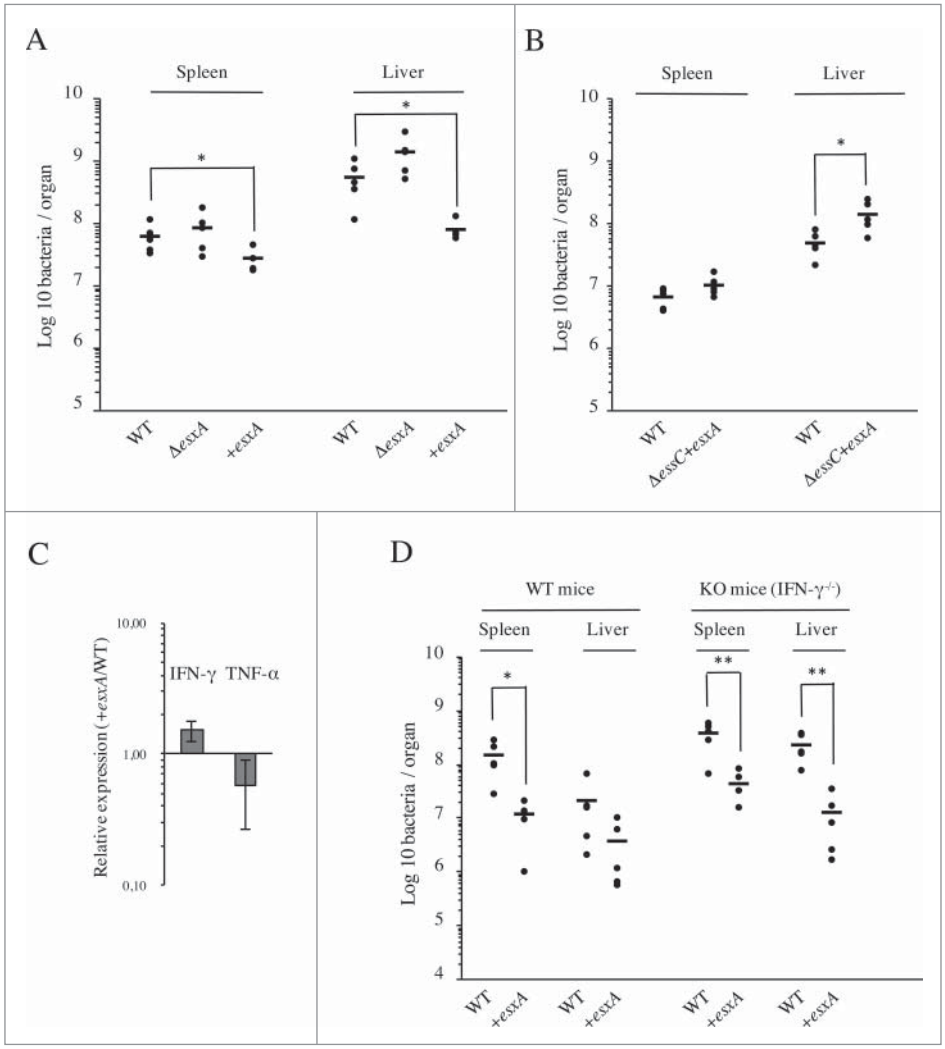

Figure 5. EsxA secretion impairs *Lm* infection *in vivo* through an IFN-γ-independent mechanism. (A) Bacterial counts for WT, Δ*esxA* and +*esxA* strains, in spleens and livers of BALB/c mice (n = 5), 72h after intravenous infection with 10^4 bacteria. (B) Bacterial counts for WT and Δ*essC*+*esxA* strains, in spleens and livers of BALB/c mice (n = 5), 72h after intravenous infection with 10^4 bacteria. (C) Levels of IFN-γ and TNF-α transcripts measured by qRT-PCR in livers of mice 72h after intravenous infection with either WT or +*esxA* bacteria. Expression levels in +*esxA*-infected livers were normalized to those infected with the WT strain. Values are mean ± SD (n = 3). (D) Bacterial counts for WT and +*esxA* strains in spleens and livers of WT and IFN-γ knock-out mice (n = 5), 72h after intravenous infection with 10^4 bacteria. *, $p \leq 0.05$; **, $p \leq 0.01$.

and *essC* and *esxC* were also shown as upregulated during cell infection.[34] Altogether, these results indicate that the ESX-1 locus of *L. monocytogenes* EGDe is poorly or not expressed in most of the conditions, with some genes of the locus expressed in few conditions that could suggest a role of the ESX-1 apparatus in these specific environments.

Using a deletion mutant and complemented strain for *essC*, we demonstrated that ESX-1 is functional in *Lm*, at least for the secretion of EsxA. Even if EssC was shown to be an essential ESX-1 element also for the secretion of EsxB in *M. tuberculosis* and *S. aureus*,[5,7] the EssC-dependent secretion of EsxB remains to be confirmed in *Lm*. EsxA and EsxB are the only WXG100 proteins predicted to be encoded by the *Lm* genome. In addition to EsxA and EsxB, the EsxC protein is also a substrate for ESX-1 in *S. aureus* and confers pathogenic function to this bacterium.[11] However, an *esxC* ortholog is absent from *Lm*. Instead, an unrelated gene of unknown function (*lmo0062*) occupies the *esxC* position in the *Lm* genome (Fig. 1). Interestingly, Lmo0062 was recently predicted to be secreted,[38] therefore investigating its capacity to be secreted in an ESX-1-dependent manner could widen the spectrum of ESX-1 substrates to proteins that do not belong to the WXG100 family.

All the above observations induced a strong presumption for the involvement of ESX-1 in *Lm* pathogenicity. However, we demonstrated that this secretion system and its substrates are not required for *Lm* cell invasion, intracellular multiplication and *in vivo* infection. In agreement with our results, EsxA

was also previously shown dispensable for *Lm* mouse infection.[23] Unexpectedly, we observed a slight increase in the infection level of mutants for ESX-1 components as compared to WT bacteria. This was also previously observed for a Δ*esxA* mutant,[23] suggesting a damaging role for ESX-1 in *Listeria* infectious capacity. In agreement with this hypothesis, overexpression of *esxA* resulted in a decrease infection of *Lm* in the mouse model, confirming the adverse effect of a functional ESX-1 secretion system. Despite our attempts to elucidate the reasons of this detrimental role, we failed to find differences regarding host immune responses upon infection by WT or *esxA* overexpressing *Lm*. ESAT-6 (the mycobacterial EsxA homolog) was shown to play a pro-apoptotic role in *M. tuberculosis*.[39] A comparable role of *Lm* EsxA could result in increased bacterial recognition and clearance by the host immune system that would explain the phenotype of *esxA* overexpressing bacteria. The absence of EssC in the bacterial membrane could also disturb cell envelope homeostasis, possibly resulting in the mislocalization of some surface proteins that could induce an increased virulence. However, no difference was observed regarding the capacity of mutants for ESX-1 components to invade or multiply inside host cells as compared to WT *Lm*.

L. monocytogenes is sensitive to a broad range of antibiotics. However, resistance to several antibiotics has been reported,[40] as well as multidrug-resistant strains.[41] The ESX-1 system could appear as a potential target for innovative anti-*Listeria* drugs that, by inducing ESX-1-dependent secretion, would be capable to impair the infectious capacity of bacteria but not their viability, significantly reducing the risk of resistance development.

Listeria has maintained this locus in its genome, suggesting that it may probably be helpful in certain conditions, such as resisting to stress encountered in specific environments. However, we were unable to find any difference between the WT and *essC* mutant regarding growth in stress conditions such as low pH (pH 5.5) and high salt concentration (4.5% NaCl) (Fig. S1C). Another reason for the conservation of this locus in the *Listeria* genome could be related to a strain issue. Indeed, we tested here the role of the ESX-1 system in only one specific *Lm* strain (*L. monocytogenes* EGDe), that is one of the most commonly used laboratory strains.[42,43] Even if EsxA was also shown to be dispensable for *in vitro* and *in vivo* growth of a different widely used *Lm* strain,[23,43] this locus could play important roles in the infection capacity of other *Listeria* strains from different serotypes.

In summary, we demonstrated here that the *Lm* genome encodes a functional ESX-1 secretion system required for the secretion of WXG100 proteins, such as EsxA. In addition, despite poorly expressed and dispensable for cell invasion, we showed that a working ESX-1 system is detrimental for *Lm* infection *in vivo*. Considering its wide distribution among Gram-positive bacteria and the lack of a convergent phenotypic trait for mutants in this pathway, ESX-1 certainly fulfils different functions that remain to be elucidated.

Materials and methods

Bacterial strains and media

Lm EGDe (ATCC-BAA-679) and *E. coli* strains were routinely cultured aerobically at 37 °C in brain heart infusion (BHI, Difco) and Lysogeny Broth (LB) media, respectively, with shaking.

The synthetic minimal medium (MM) was prepared as previously described.[44] 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.

Construction and complementation of mutant strains

lmo0056, lmo0061 and *lmo0063* deletions were performed in the EGD-e background through a process of double homologous recombination mediated by the suicide plasmid pMAD as described[45] using the corresponding oligonucleotides (A-D; Table S1). Genetic complementation of the deletion mutant strains was performed using the phage-derived integrative plasmid pPL2 as described[45] using the respective oligonucleotides (Table-S1). For overexpression, target genes were cloned into the pRB474 vector as described.[46] All plasmid constructs and strains were confirmed by PCR and DNA sequencing.

Western blot analysis of EsxA

Overnight bacterial cultures grown in static conditions (final OD_{660} ~1.0) were centrifuged (10 000 g, 10 min, 4°C) and the supernatant filtered using Millipore 0.45 μm filters. A volume of 1.6 ml of 50% trichloroacetic acid was added to 6 ml of the filtered supernatant and incubated for 1 h at 4°C. The sample was centrifuged (30 000 g, 20 min, 4°C) and the pellet washed with cold acetone, repeating the centrifugation step in same conditions. The pellet was dried, suspended in 30 μl of PBS pH 7.4 and mixed with 15 μl of 4x Laemmli buffer. A volume of 15 μl was loaded in a 12% SDS-polyacrylamide gel. For

total bacterial lysates, the bacterial pellet from 10 ml of culture was suspended in 0.8 ml of PBS pH 7.4 containing 100 μg/ml DNase and protease inhibitors. Bacteria were lysed in a FastPrep-24 homogenizer (MP Biomedicals) (30 s, maximum speed) and cell debris removed by centrifugation (3 000 g, 5 min, 4°C). A volume of 40 μl of 4x Laemmli buffer was added to 100 μl of supernatant of bacterial lysates, and 10 μl loaded into the gels. Western blotting was performed as described[46] using anti-Myc tag mouse antibody (#clone 9B11, Cell Signaling #2276).

Gene expression analyses

Bacterial RNAs were isolated from 10 ml of cultures at the desired growth phase. For quantification of cytokine expression in mouse livers, organs were homogenized in RNAlater stabilization solution (Qiagen), quick-frozen in dry ice and stored at -80°C. Total RNAs were extracted by the phenol-chloroform method as previously described,[47] 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). 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 primers (Table S1), using the following protocol: 1 cycle at 95 °C (5 min), 25 cycles at 95 °C (30 s), 55 °C (30 s) and 72 °C (20 s), and 1 cycle at 72 °C (5 min). Amplification products were resolved in 1% (w/v) agarose gel and analyzed in a GelDoc XR+ System (Bio-Rad Laboratories). Quantitative real-time PCR (qRT-PCR) 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 S1), using the following cycling protocol: 1 cycle at 95 °C (3 min) and 40 cycles at 95 °C (30 s), 55 °C (30 s) and 72 °C (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 ($\Delta\Delta$Ct) method, after normalization of the test and control sample expression values to a housekeeping reference gene (16S rRNA).

Adhesion and invasion assays

Adhesion and invasion assays were performed as described.[45] Briefly, Caco-2 (ATCC, HTB-37) and Vero (ATCC, CCL-81) cells were seeded (in triplicate) per 24-well plates (~2 × 10^{55}/well) in EMEM 20% foetal bovine serum and DMEM 10% foetal bovine serum, respectively (LONZA), and propagated for 48 h. *Listeria* were grown in BHI to OD_{600nm} = 0.8, washed and inoculated at 50 bacteria-per-cell for 1h. For adhesion, cells were washed 3 times, lysed in 0.2% Triton X-100 and viable bacteria were enumerated after plating serial dilutions of the lysates in BHI agar media. For invasion assays, cells were infected for 1h and treated with 20 μg/ml gentamicin for 1h30 before lysis in 0.2% Triton X-100.

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.[37] Briefly, cells (~2×10^{55}/well) were infected for 45 min with exponential-phase bacteria at ~10 bacteria/cell 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.

Animal infections

Infections were performed in 6-to-8 week-old specific-pathogen-free females as described.[48] Briefly, wild-type BALB/c (Charles River Laboratories) or wild-type IFN-γ knockout C57BL/6J mice were infected intravenously with 10^4 CFUs in PBS, or starved 12h before gavage inoculation with 10^9 CFUs in PBS containing 150 mg/ml $CaCO_3$. The infection was carried out for 72 h, at which point the animals were euthanized 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. 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*.

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.

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 2 groups; one-way ANOVA was used with Tukey's post-hoc test for pairwise comparison of means from more than 2 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: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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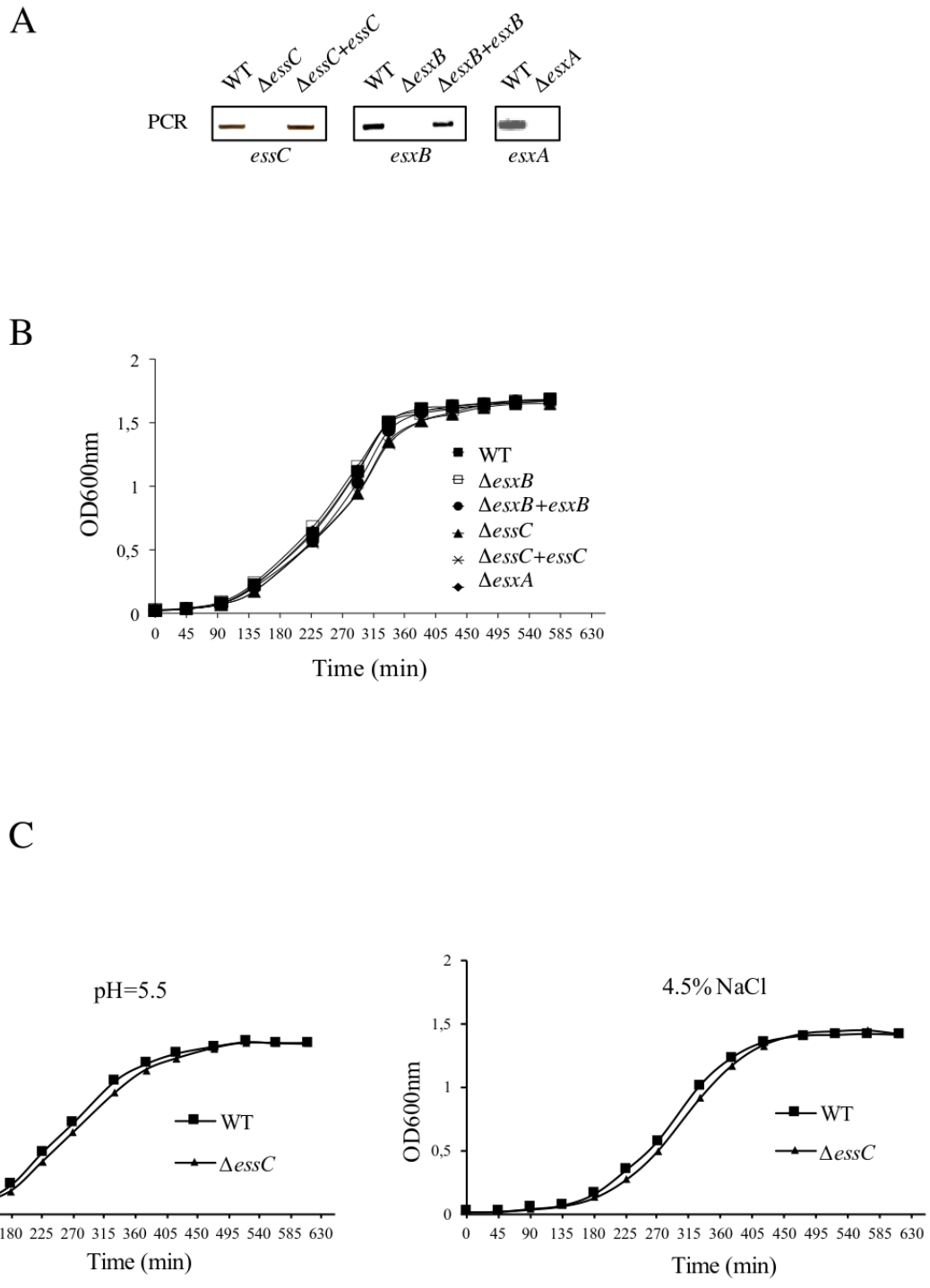
A
WT
ΔessC
ΔessC+essC
WT
ΔesxB
ΔesxB+esxB
WT
ΔesxA
PCR
essC
esxB
esxA
B
OD600nm
Time (min)
WT
ΔesxB
ΔesxB+esxB
ΔessC
ΔessC+essC
ΔesxA
C
pH=5.5
4.5% NaCl
OD600nm
Time (min)
WT
ΔessC

Figure S1

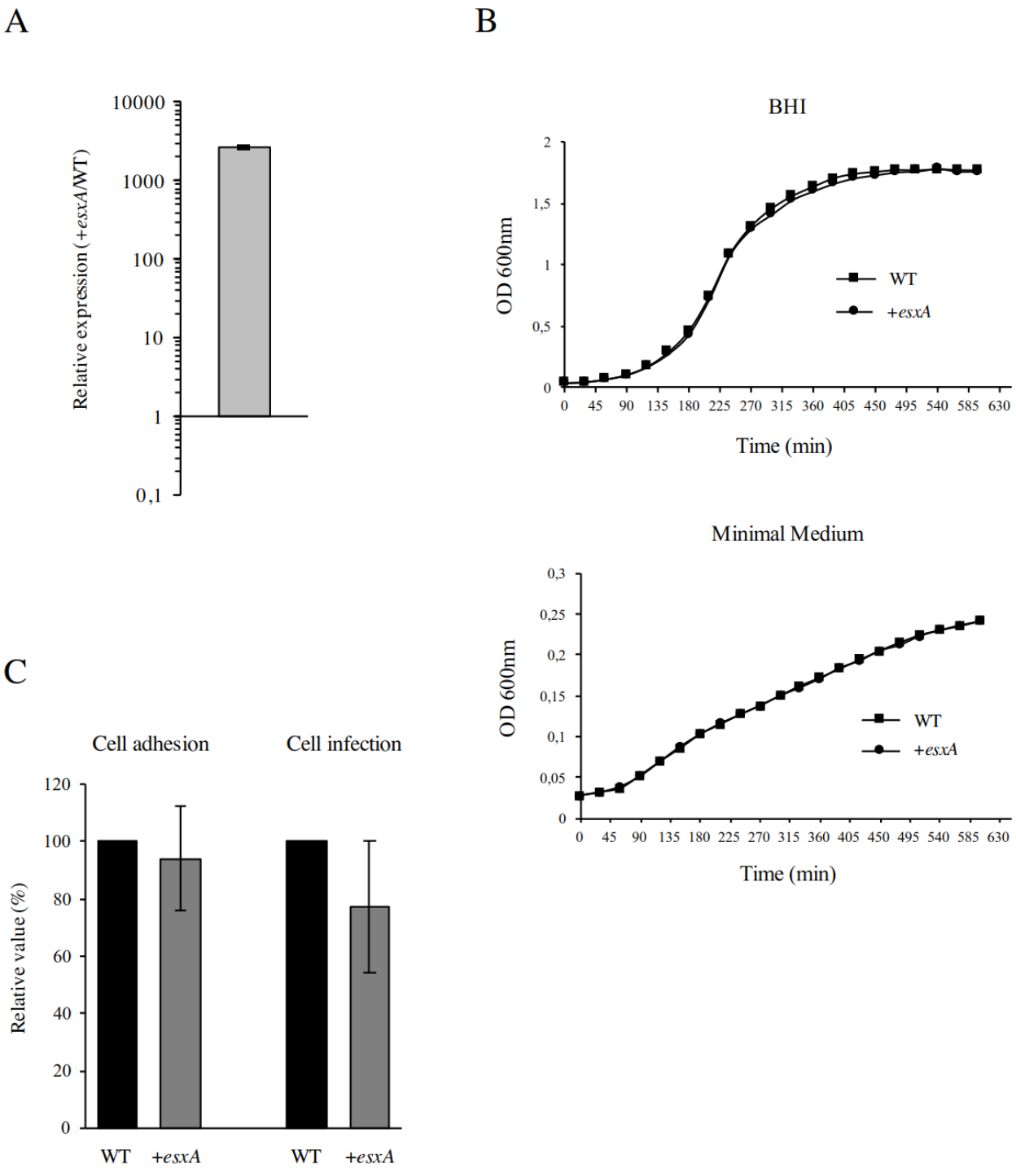
A
B
C
10000
1000
100
10
1
0,1
Relative expression (+esxA/WT)
BHI
OD 600nm
Time (min)
WT
+esxA
Minimal Medium
Cell adhesion
Cell infection
Relative value (%)
120
100
80
60
40
20
0
WT
+esxA

Figure S2

Figure S1

(A) Genotype confirmation of *esxA, esxB* and *essC* deletion mutant and corresponding complemented strains by PCR. (B) Growth curves of WT, Δ*esxA,* Δ*esxB,* Δ*esxB*+*esxB,* Δ*essC* and Δ*essC*+*essC* strains at 37°C in BHI broth. (C) Growth curves of WT and Δ*essC* strains at 37°C in BHI broth with pH adjusted to 5.5 or supplemented with 4.5% NaCl.

Figure S2

Phenotypic analysis of the *Lm* strain overexpressing *esxA* (+*esxA*). (A) The expression level of *esxA* in the +*esxA* strain was measured by qRT-PCR and is shown relative to its level in WT strain. Values are mean ± SD (n=3). (B) Growth curves of the WT and +*esxA* strains at 37°C in BHI broth and minimal medium. (C) Caco-2 cell adhesion and invasion by the WT and +*esxA* strains. Values are mean ± SD (n=3) and are expressed as percentage relative to WT values arbitrarily fixed to 100%.

Table S1 - Oligonucleotides

Name	Sequence (5' > 3')
lmo0063-A	atgtggatcctgaactaattggtcagaatatgg
lmo0063-B	gattacgcgttctttagcctctcctctcgttc
lmo0063-C	ttacgcgtcagtggaaacgatagacgtattttca
lmo0063-D	caagagatcttcatagtcttttcacatccttgg
lmo0056-A	cggatcccgctttagatgatgg
lmo0056-B	gtacgcgtcattcacttcatcacc
lmo0056-C	cgacgcgttaaaatcaacaaaaagtgg
lmo0056-D	cagagatctaagttttctgtgaattctgc
lmo0061-A	caggtcgacacaagttcgaaaaatgggacg
lmo0061-B	agacgcgtggtccattctcccttattttgc
lmo0061-C	cgacgcgttgaaaggagctgttaatgatgg
lmo0061-D	ggaattctcattgggtgtttttttctag
lmo0056-RT-PCR Fw	ggtcaaattcgtatgagtcca
lmo0056-RT-PCR Rv	atcgtgctcttctactgcgtttgc
lmo0058-RT-PCR Fw	gggagttctcgctgcggattcag
lmo0058-RT-PCR Rv	aagtgagtctttaatccggtca
lmo0060-RT-PCR Fw	cttgtacaatggctcgctatcagg
lmo0060-RT-PCR Rv	agcgacgataatgaaaccaaccg
lmo0061-RT-PCR Fw	tgggcatttagctgtgttttctagtc
lmo0061-RT-PCR Rv	attctagtgttaagcgacgaataag
lmo0063-RT-PCR Fw	ggtaatgtgaaaattgatgccgc
lmo0063-RT-PCR Rv	gagtatgaagtattctcaaaatcc

CHAPTER IV – GENERAL DISCUSSION

General Discussion

In this thesis, we reported studies of novel advancements that contribute to the enlightening and better understanding of the regulation of virulence in *Lm*. Pathogenic bacteria face the challenge of constant modulation of their physiology in order to adapt to changes in the environment and their host (Freitag et al., 2009). Bacteria achieve this through complex regulation of their genomes, ensuring an optimal gene expression profile but in particular the expression of virulence factors. This process of adaptation includes changes in the composition of the cell surface and in the secretion of proteins (Carvalho et al., 2014). We demonstrated here the roles of two novel *Lm* transcription regulators MouR and Lmo0443 and of the ESX-1 bacterial secretion system in the virulence regulation of *Lm*.

Unraveling novel virulence determinants in genomes comprising thousands of genes can be a challenging task. Array- and sequencing-based high throughput genomics and transcriptomics have been intensively used in the past years as powerful tools in genome-wide characterization of bacterial gene composition and gene expression. In this work, we have demonstrated how we successfully employed a combination of high throughput data with *in silico* analyses to identify and characterize novel *Lm* virulence regulators. Among hundreds of putative transcription factors predicted to be encoded in the genome of *Lm* EGD-e, we were able to pinpoint the regulators MouR and Lmo0443 and demonstrate their implication in virulence. Indeed, genes responsible for encoding these regulators were found to be overexpressed during the infection of mouse spleens when compared to growth in BHI (Camejo et al., 2009). This reflects how the expression of many genetic elements which are important for the adaptation of *Lm* to the host environment is augmented during infection. Our studies of MouR and Lmo0443, which included additional genome-wide high throughput transcriptomic approach, contributed both to the characterization of specific regulatory proteins and mechanisms as well as to the general understanding of the *Lm* virulence transcriptional regulatory network organization.

Transcription factors are a major component of the bacterial gene regulation machinery, that typically work by binding to DNA promoter regions upstream of the coding sequencing of their target genes. How these proteins function and how they regulate gene expression is intrinsically related to their general protein structure, to their conformation (secondary, tertiary, quaternary conformations), to their DNA-binding properties and to their interaction with other proteins and other molecules (Sai et al., 2011). However, despite the enormous amounts of transcription factor entries in databases like Pfam, still only a small fraction of these include crystal structure data and proper protein characterization. In this thesis, we contribute to the study of these class of proteins by resolving the crystal structure of MouR, a novel VanR class

member of the FadR family of GntR transcription regulators. By resolving its tri-dimensional structure, we were able to demonstrate in detail how MouR monomers form stable dimers by interaction of their C-terminal oligomerization domains and how their N-terminal has a domain capable of specifically binding the promoter DNA of the Agr system, activating its expression. The availability of MouR crystal structure data can lead the way to interesting follow up studies such as the identification of possible interactions between the MouR C-terminal with other proteins or molecules (e.g. as a stimuli sensing-like mechanism) that could modulate MouR activity, adding new layers to this *Lm* virulence regulation network. Importantly, this could also be useful to many other studies dedicated to the characterization of similar proteins that could resort to MouR crystal data for superimposition/modeling purposes.

This work also elucidated an important missing link between the Agr system and *Lm* virulence. Whereas the well-studied *S. aureus* counterpart Agr system has several positive and negative regulators controlling its expression (Thoendel et al., 2011), to our knowledge no regulator of the *agr* genes had been characterized in *Lm*. Hence, we provided for the first time the characterization of a central activator of the *Lm* Agr system. Mechanisms of virulence regulation can rely on complex networks of gene control characterized by multiple layers. We have shown here how MouR introduces a new layer on a complex cascade of regulatory events that include classical transcription factor binding to promoter DNA sequences but also sRNA-mediated regulation, that culminate in enhanced survival and virulence of *Lm*. In fact, the implication of Agr in diverse biological roles such as survival and competition in soil (Vivant et al., 2014, 2015), biofilm formation (Riedel et al., 2009) and chitinase-mediated host immune-modulation (Chaudhuri et al., 2013; Nielsen et al., 2011) indicates that MouR has a versatile role aside from virulence. We hypothesize that this wide range of functions could be one reason why this virulence regulator is frequent and fairly conserved even among non-pathogenic *Listeria* species. Overall this study provided relevant insights about how *Lm* tunes the expression of this important system and regulates virulence. Elucidation about the implication of MouR specifically during the gastric phase of infection requires further investigation.

In this work, we also demonstrated the role of another *Lm* putative LytR-LCP transcription factor Lmo0443 in virulence. We observed how the absence of its encoding gene results in attenuated virulence *in vivo*. By a genome-wide transcriptomic approach we determined that Lmo0443 represses transcription of *lmo0444,* a gene belonging to the stress tolerance islet SSI-1 (Ryan et al., 2010). We demonstrated how further phenotypical analysis of a mutant mimicking the lack of Lmo0443 repression (by *lmo0444* overexpression) was compromised by a serious defect generated in growth and cell morphology. While it remains mostly unclear how Lmo0444 could modulate *Lm* virulence by upstream control of Lmo0443, our studies suggest that this could be closely related to regulation of cell dynamics and composition in surface

proteins. Although we believe that a deeper characterization of both Lmo0443 and Lmo0444 would require further investigation, this work has provided a contribution to unraveling the novel roles of Lmo0443 in virulence and also suggests for the first time a possible role for the uncharacterized SSI-1 component Lmo0444 in cell envelope dynamics.

In this thesis we also report a novel description of *Lm* virulence regulation at the level of protein secretion. We demonstrated how the ESX-1 secretion system, thought to be dispensable for *Lm* infection (Way and Wilson, 2005), could have a detrimental role for *Lm* virulence *in vivo*. Our work shows how the tight regulation of surface and secreted proteins can be crucial for successful infection of pathogenic bacteria. These novel data regarding the role of the ESX-1 system raises questions about the motive for *Lm* to keep the *esx* locus on its genome throughout evolution and sustain the energetic burden associated with its expression and having its expression and function under control. We hypothesize that the reason behind this could be the possibility of alternative roles of the ESX-1 in the adaptation of *Lm* to different types of environment. Due to its very robust physiology *Lm* can survive in diverse types of environment outside the host (e.g. soil, water, animal feed, refrigerated foods, etc.) and it is possible that ESX-1 can have specific roles that favor *Lm* in these specific environments.

In conclusion, we believe that the work reported here constitutes a relevant contribution to the current knowledge on the *Lm* virulence regulation scene. A better understanding of virulence regulation is a very important aspect in the context of the *Lm* infectious process which, as a model, is of high relevance for the broader field of bacterial pathogenesis. We provided novel characterization of three independent virulence regulatory mechanisms, two transcription factors and a protein secretion system. Importantly, further work should focus on the specific conditions and/or molecular stimuli that induce or modulate all three regulatory systems. We also believe that the kind of approaches reported here should continue to be employed in the identification of novel *Lm* virulence regulators.

CHAPTER V – REFERENCES

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Curriculum Vitae

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Education

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Institute of Molecular and Cellular Biology / i3S Institute for Research and Innovation in Health / Institute of Biomedical Sciences Abel Salazar, University of Porto, Portugal
PhD student in Biomedical Sciences.

2009/2011

Biology Department, University of Aveiro, Portugal
Master's Degree in Applied Biology – Clinical and Environmental Microbiology. Final mark of 18 out of 20 (A class in the ECTS scale).

2010/2011

Biology Department, University of Aveiro, Portugal
Master's thesis project for the degree of Master in Applied Biology – Clinical and Environmental Microbiology. Project based on the study of the potential of an ash tree in the phytoremediation of metals and the study of the variations on the bacterial (endophytic and rhizosphere) communities associated to the roots of the plants. Work developed with the support of NATO under the project SfP 983311 - "Processes for the remediation of uranium mines and other minerals". Experimental work reported in a dissertation entitled "Metal effects in *Fraxinus angustifolia* and its endophytic communities". Final mark 20 out of 20.
Supervisors: Prof. Dr. Ruth Maria de Oliveira Pereira and Dr. Catarina Pires Ribeiro Ramos Marques.

2006/2009	**School of Sciences, University of Minho, Portugal** First degree in Applied Biology. Final mark of 16 out of 20 (B class in the ECTS scale).
May 2009/July 2009	**Biodiversity Laboratory, Biology Department, University of Minho, Portugal** Final year project, for the degree of Applied Biology. Experimental work reported in a dissertation under the theme "Confocal microscopy for the study of virus vector nematodes, genus *Trichodorus* (Nematoda: Trichodoridae)". Final mark 19 out of 20. Supervisor: Prof. Dr. Maria Teresa da Silva Craveiro Martins Almeida.

Previous and Current Activities

November 2012 – Present	**Molecular Microbiology Group – IBMC-Porto, Portugal** Worked as a fellow researcher and currently as a PhD student in the Molecular Microbiology Group at IBMC-Porto under the supervision of Didier Cabanes.
2010 – 2011	**Biology Department, University of Aveiro, Portugal** Master's degree researcher in the LEADER laboratory. Study of the phytoremediation of metals and the potential of an ash tree species (*Fraxinus angustifolia*) as a candidate for the process. Study of the effects of metals on the diversity and structure of endophytic and rhizosphere bacteria associated with the roots of *F. angustifolia*.
May 2009 – July 2009	**Biodiversity Laboratory, Biology Department, University of Minho, Portugal** Researcher of the final year project in the Biodiversity Laboratory in the experimental work reported in a dissertation entitled "Confocal microscopy for the study of virus vector nematodes, genus *Trichodorus* (Nematoda: Trichodoridae)".
April 2009	**Biology Department, University of Minho, Portugal** Volunteer as experimental worker for the PhD project of José Carlos Carvalho, University of Minho (under the supervision of Prof. Dr. Pedro Miguel Bondoso Cardoso and Prof. Dr. Pedro Alexandre Faria Fernandes Teixeira Gomes).

Publications

Thesis

Master's Degree Thesis – Universidade de Aveiro 2011
Jorge Nuno Martins Campos Pinheiro (2011). Metal effects in *Fraxinus angustifolia* and its endophytic communities

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Peer-Reviewed International Journal Publication

Pinheiro, J., Reis, O., Vieira, A., Moura, I., Moreno, L., Carvalho, F., Pucciarelli, M., García-del Portillo, F., Sousa, S., Cabanes, D. **(2017)** *Listeria monocytogenes* encodes a functional ESX-1 secretion system whose expression is detrimental to in vivo infection. Virulence, 8, 993–1004.

Pombinho, R., Camejo, A., Vieira, A., Reis, O., Carvalho, F., Almeida, T., **Campos Pinheiro, J.**, Sousa, S. and Cabanes, D. **(2017)** *Listeria monocytogenes* CadC regulates cadmium efflux and fine-tunes lipoprotein localization to escape the host immune response and promote infection. The Journal of Infectious Diseases. 215, 1468-79.

Mesquita, F., Brito, C., Moya, M., **Pinheiro, J.**, Mostowy, S., Cabanes, D., Sousa, S. **(2016)** Endoplasmic reticulum chaperone Gp96 controls actomyosin dynamics and protects against pore-forming toxins. EMBO reports - DOI 10.15252/embr.201642833

Pinheiro, J. C., Marques, C. R., Pinto, G., Bouguerra, S., Mendo, S., Gomes, N. C., Gonçalves, F., Rocha-Santos, T., Duarte, A. C., Roembke, J., Sousa, J. P., Ksibi, M., Haddioui, A., Pereira, R., **(2013)**. The performance of *Fraxinus angustifolia* as a helper for metal phytoremediation programs and its relation to the endophytic bacterial communities. Geoderma, 202-203, 171-182.

Duarte, K. R., Freitas, A. C., Pereira, R., **Pinheiro, J. C**., Gonçalves, F., Azaari, H., Azzouzi, M. E:, Zrineh, A., Zaydoun, S, Duarte, A. C., Rocha-Santos, T. A., **(2012**). Treatment of Olive Oil Mill Wastewater by Silica–Alginate–Fungi Biocomposites. Water, Air, & Soil Pollution, 223 (7), 4307.

Poster

Title: LarS controls the expression of the *agr* system of *Listeria monocytogenes* and mediates virulence
Authors: **Jorge Pinheiro**, Johnny Lisboa, Filipe Carvalho, Alexis Carreaux, Nuno Santos, Sandra Sousa and Didier Cabanes
Congress: 7th Congress of European Microbiologists - FEMS 2017
Venue/Date: Valencia, Spain – 2017

Title: Molecular characterization of newly identified Listeria monocytogenes virulence regulators
Authors: **Jorge Pinheiro**, Alexis Carreaux, Filipe Carvalho, Sandra Sousa and Didier Cabanes
Congress: EMBO Conference: Problems of Listeriosis ISOPOL XIX
Venue/Date: Institut Pasteur Paris, France – 2016

Title: Molecular characterization of newly identified Listeria monocytogenes virulence regulators
Authors: **Jorge Pinheiro**, Alexis Carreaux,Filipe Carvalho, Sandra Sousa and Didier Cabanes
Congress: V I3S Scientific Retreat.
Venue/Date: Axis Vermar Hotel, Póvoa de Varzim, Portugal – 2016

Title: Molecular characterization of newly identified Listeria monocytogenes virulence regulators
Authors: **Jorge Pinheiro**, Filipe Carvalho, Sandra Sousa and Didier Cabanes
Congress: EMBO | EMBL Symposium: New Approaches and Concepts in Microbiology
Venue/Date: EMBL Heidelberg, Germany - 2015

Title: Metal effects in Fraxinus angustifolia and its endophytic communities
Authors: **Pinheiro, J.**; Marques, C. R.; Pinto, G.; Mistiri, A.; Mendo, S.; Gomes, N. C. M.; Gonçalves, F.; Rocha-Santos, T.; Duarte, A. C.; Römbke, J.; Sousa, J. P.; Ksibi, M.; Haddioui, A.; Pereira, R. (Presenter)
Congress: 22st SETAC Europe Annual Meeting
Venue/Date: Estrel hotel – Berlin, Germany - 2012

Supervision Experience

Co-supervision of master student Alexis Carreaux (Université Paris Diderot) in the context of his "Master 1 - Infectiology Microbiology Virology and Immunology" Erasmus internship.

Courses

2013 **Postgraduate Course:** Next Generation Sequencing: approaches and applications. ICVS – Universidade do Minho, Braga, Portugal.

2012 **FELASA Category C Certificate:** Course in Laboratory Animal Science - Category C, Accredited by FELASA. IBMC – Universidade do Porto, Porto, Portugal.

2008 **Practical Course:** I Practical Course in Immunology. Department of Biology, University of Minho, Braga, Portugal.

Non-Academic experience

2008/2009 Scientific event organization experience as a member of the organizing committee of the X Meeting of Applied Biology, University of Minho, Braga.

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Language Abilities

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