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ADDENDUM

Listeriolysin S: A bacteriocin from epidemic *Listeria monocytogenes* strains that targets the gut microbiota

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ABSTRACT

Listeria monocytogenes is a Gram-positive food-borne pathogen that in humans may traverse the intestinal, placental and blood/brain barriers, causing gastroenteritis, abortions and meningitis. Crossing of these barriers is dependent on the bacterial ability to enter host cells, and several *L. monocytogenes* surface and secreted virulence factors are known to facilitate entry and the intracellular lifecycle. The study of *L. monocytogenes* strains associated to human listeriosis epidemics has revealed the presence of novel virulence factors. One such factor is Listeriolysin S, a thiazole/oxazole modified microcin that displays bactericidal activity and modifies the host microbiota during infection. Our recent results therefore highlight the interaction of *L. monocytogenes* with gut microbes as a crucial step in epidemic listeriosis. In this article, we will discuss novel implications for this family of toxins in the pathogenesis of diverse medically relevant microorganisms.

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Introduction bacteriocins

Bacteriocins are ribosomally-synthesized peptides or proteins which have been reported in bacteria and archea, which can kill species closely related to the producer species, and for which the producer often displays an immunity mechanism.[1] Historically, the term 'bacteriocin' coined by François Jacob in 1953[2] referred to colicins produced by *Escherichia coli*[3] and analogous proteins produced by other Gram-negative bacteria including pyocins from *Pseudomonas pyocyanea*,[4] marcenins from *Serratia marcescens*, cloacins produced by *Enterobacter cloacae* or influenzacins by *Haemophilus influenzae*.[5] In Gram-positive bacteria, bacteriocin-like activities were described as early as 1928[6] but the name 'bacteriocin' was used only in later years,[7] in particular as a reference to lantibiotics and other peptides produced mainly by lactic acid bacteria.[8]

Several bacteriocin classification schemes have been proposed, which either include only molecules produced by Gram-positive bacteria[9] or molecules produced by both Gram-positive and Gram-negative bacteria.[10] Nowadays, the term 'bacteriocin' comprises diverse molecules which include circular peptides, non-modified peptides and post-translationally modified peptides, including members of the thiazole/oxazole modified microcins (TOMMs). The prototypic TOMM is Microcin B17 from *Escherichia coli*,[11] a DNA gyrase inhibitor that displays 14 post-translational modifications constituted of thiazole and oxazole rings.[12] Among the TOMMs there are also molecules for which no bacteriocin activity has been reported: for example the *Streptococcus pyogenes* TOMM Streptolysin S (SLS) is a toxin which displays hemolytic activity, and cytotoxic activity against macrophages and neutrophils. SLS is also involved in paracellular invasion of tissues and in soft tissue damage, but has no reported bacteriocin activity.[13]

It has been proposed that 99% of all bacteria may produce at least one bacteriocin.[14] Interestingly, we

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have recently reported the first bacteriocin produced for the Gram-positive genus *Listeria*.[15]

Listeria monocytogenes: *A food-borne pathogen*

Listeria monocytogenes is a major bacterial model system in diverse research fields including microbiology, immunology and cell biology. This Gram-positive pathogen is responsible for listeriosis, a food-borne disease characterized by potentially fatal septicemia in immunocompromised individuals, severe meningitis in newborns and abortion in pregnant women. Considered for many years as a rare bacterium after its first descriptions in England in 1926[16] and in South Africa in 1927,[17] *L. monocytogenes* has been instrumental in our understanding of cellular immune responses since the pioneering work of George Mackaness in the 1960s,[18] which demonstrated that this bacterium is able to multiply within macrophages and established it as the prototype intracellular parasite. The important listeriosis epidemics in North America and Europe in the 1980s subsequently revealed that *L. monocytogenes* also represents an important public health problem,[19] being responsible for the largest and most deadly food recalls in the United States.[20] In the following years, critical aspects of *L. monocytogenes* virulence mechanisms were discovered due to advances in molecular and cell biology techniques: for example, the pore-forming toxin listeriolysin O (LLO), which allows *L. monocytogenes* escape from phagosomes, became the first bacterial virulence factor to have its gene cloned.[21] Soon afterwards, the intracellular cycle of *L. monocytogenes* was discovered,[22] highlighting that this bacterium manipulates the actin cytoskeleton to spread from cell to cell,[23] allowing its use as a molecular tool to identify the Arp2/3 complex as the first discovered actin nucleator in eukaryotic cells.[24] Since then, *L. monocytogenes* has been studied as a model pathogen,[25] as an exquisite tool to manipulate mammalian cells for the identification of novel cellular functions,[26,27] and as vector to deliver intracellular antigens in anticancer therapies.[28]

The *L. monocytogenes* pathogenicity island I (LIPI-I) and the internalins islet encode for bacterial molecules which modulate host cells functions (reviewed by,[25,29]): these include the surface proteins InlA and InlB which promote bacterial internalization into host cells,[30] the cholesterol-dependent cytotoxin LLO and the phospholipases PlcA and PlcB which disrupt host cell membranes,[21,31] and the surface protein ActA involved in host actin polymerization.[23] Two additional genes in LIPI-I encode for Mpl, a metalloprotease involved in the maturation of PlcB[32] and for PrfA, the major transcription factor which controls the expression of the most important *L. monocytogenes* virulence genes.[33] It is important to note that for decades, *L. monocytogenes* pathogenesis has been mainly studied using a subset of bacterial strains including EGD, EGDe, LO28 and 10403S which belong to the *L. monocytogenes* evolutionary lineage II, an assembly of bacterial clonal groups which are rarely associated to major human listeriosis outbreaks.[34,35] On the other hand, listeriosis epidemics in humans are mostly associated to *L. monocytogenes* clonal groups from the evolutionary lineage I, but these strains have been poorly characterized and the molecular mechanisms that contribute to their higher virulence remained unknown until recent times.[35] In 2008, the *L. monocytogenes* pathogenicity island III (LIPI-III) was discovered in a subset of lineage I strains, suggesting that it could be associated to the higher virulence potential of these bacteria.[36] LIPI-III encodes a biosynthetic cluster involved in the production of Listeriolysin S (LLS), an hemolytic and cytotoxic TOMM shown to be required for *L. monocytogenes* virulence *in vivo* in a mouse intra-peritoneal infection model.[36]

Listeriolysin S: A bacteriocin that modulates the host microbiota

The LLS gene cluster include the gene *llsA* which encodes for the actual LLS toxic peptide, the genes *llsG* and *llsH* which encode for a putative transporter, the genes *llsB, llsY* and *llsD* which encode for putative post-translational modification enzymes involved in the production of thiazole/oxazole/methyloxazole rings, the gene *llsP* which encodes for a putative protease, and the gene *llsX* which encodes for a protein of unknown function specific to the genus *Listeria*.[36,37] Inactivation of the *llsB* gene was sufficient to reduce *L. monocytogenes* numbers in the liver and spleen *in vivo*, suggesting that the post-translational modification of the *llsA* gene product is crucial for its biologic activity.[36]

To better understand the potential contribution of LLS to *L. monocytogenes* virulence, we decided to use a mouse oral infection model, which recapitulates the

normal infection route in humans for this food-borne pathogen. We first compared infection by 2 lineage II strains, EGDe and 10403S, with the lineage I strain F2365 responsible for the 1985 listeriosis California outbreak.[38] Our results indicate that the lineage I strain is more virulent than the 2 lineage II strains after counting colony forming units in the intestinal content, in the intestine and in the spleen 48 hours after infection.[15] To specifically evaluate the role of LLS in this oral infection model, we generated deletions mutants for the *llsA* and *llsB* genes: surprisingly, we observed for these mutants an important reduction in their capacity to invade the intestine and to survive in the intestinal content as early as 6 hours post-infection, suggesting that the LLS could play a role in virulence during the *L. monocytogenes* intestinal stage.[15] To monitor the organs in which LLS is expressed *in vivo*, we fused the promoter region of the LLS gene cluster to the *lux* operon of *Photorhabdus luminescens*: we detected the production of bioluminescence specifically in the intestine of orally-infected mice from 7 hours post-infection (the first post-infection measurement of bioluminescence in our experimental conditions). Upon dissection of animals at 96 hours post-infection, bioluminescence was detected only in the intestine and not in other infected organs including the liver and the spleen (although these organs contained higher bacterial counts), suggesting a major role for LLS in the mouse intestine.[15]

The biosynthetic cluster encoding LLS is homologous to the operon encoding the microcin B17 from environmental *E. coli*, which is a bacteriocin that kills competitor soil bacteria. As *L. monocytogenes* encounters diverse bacterial communities during the intestinal phase of listeriosis, we decided to explore the hypothesis that LLS could behave as a bacteriocin and influence virulence by modulating the host gut microbiota. First, we investigated *in vitro* the capacity of LLS to alter the growth of potential target bacteria by incubating a F2365 lineage I strain constitutively producing LLS with the 10403S and EGD lineage II strains which do not possess the LLS biosynthetic cluster, and which therefore lack the putative immunity gene *llsP*. Our results clearly indicate that the production of LLS is associated with a decrease in the survival of the lineage II strains, suggesting that LLS displays bacteriocin activity. Moreover, a screen of a small library of Gram-positive and Gram-negative bacteria indicate that LLS is only active against other Firmicutes which include *Lactococcus lactis* and *Staphylococcus aureus*.[15] In the light of these results, we decided to monitor changes in the host intestinal microbiota associated to LLS production using high-throughput 16S rDNA analysis. We infected mice with a F2365 wild type strain, with an isogenic deletion mutant of the *llsA* gene or with an isogenic complemented strain, and we examined the microbial community compositions 24 hours after infection. Our results show that LLS production does not promote major changes in the host microbiota at the phylum level: instead, significant changes are detected only at the genus level for representatives of *Alloprevotella, Allobaculum* and *Streptococcus* in mice infected with the wild type and the complemented strains, but not in mice infected with the *llsA* deletion mutant.[15]

In summary, our study reports the first bacteriocin for the *Listeria* genus (Fig. 1). For decades, *L. monocytogenes* had been one of the most studied food-borne bacterial pathogens, from an infection biology perspective. However, investigations focused on lineage II strains have prevented the identification of novel virulence factors exclusively associated to lineage I strains. Our findings pave the way to understand why lineage I *L. monocytogenes* strains are more often associated to human listeriosis outbreaks, and in particular they suggest that modulation of the host microbiota is critical for the infection outcome. Whether bacteriocin production by epidemic entero-pathogenic bacteria is a common strategy to colonize the gastrointestinal tract, or whether it is an exceptional mechanism used only by some *L. monocytogenes* and *Enterococci* strains during their infection processes, remains to be elucidated.[39]

Open questions

Cellular activities

LLS is a member of the TOMM family which has been described previously as an hemolytic and cytotoxic factor.[36] Now, we report LLS behaving also as bacteriocin[15] This dual feature is not specific to LLS as it has been previously observed in a molecule secreted by *Staphylococcus pseudointermedius*: indeed, the peptide BacSp222 displays both features of a cytotoxic factor against eukaryotic cells and a bacteriocin toward Gram-positive bacteria.[40] Our work clearly indicates that the bacteriocin activity of LLS plays a crucial role during infection. Previous results

from Cotter et al. suggest that the cytotoxic activity of LLS also contributes to *L. monocytogenes* virulence.[36] Using our bioluminescent reporter, we were not able to detect the activation of the LLS promoter in other organs besides the intestine.[15] However, we cannot exclude that activation of the LLS operon takes place at levels which are not detected by our reporter system. It remains to be determined which cellular populations are targeted by LLS *in vivo* (Fig. 2): it would be tempting to speculate that LLS may either favor vacuolar escape (in cooperation with LLO, PlcA and PlcB) or that its hemolytic role may favor *L. monocytogenes* survival in the blood.

Bactericidal mechanism

The mechanism by which LLS achieves bacterial killing is unknown. Several lantibiotics use as receptors the lipid II enzyme involved in the translocation of peptidoglycan subunits from the bacterial cytoplasm to the cell wall, and these lantibiotics inhibit therefore cell wall synthesis (reviewed by[13]). Nisin is a particular bacteriocin that binds lipid II and it blocks not only lipid II activity but it can also insert into the bacterial inner membrane inducing the formation of pores and consequently promoting bacterial killing by disrupting ion gradients.[41] On the other hand, the prototypic TOMM microcin B17 uses specific outer membrane and inner membrane transporters to reach the cytoplasm of Gram-negative bacteria, where it inhibits the activity of the DNA gyrase.[12] Whether LLS displays a pore-forming activity, an enzymatic/nuclease activity or both therefore remains to be identified. Determination of the mature structure of LLS may provide clues on its bactericidal mechanism. The mature structures of Gram-negative TOMMs, including the microcin B17, have been identified.[12] Interestingly, the structures of TOMMs from Gram-positive bacteria have proven to be more difficult to solve: despite more than 100 y of investigation on SLS, the structure of its mature form is still unknown.[42] Recent advances in proteomic approaches, coupled to targeted site mutagenesis studies, could allow the identification of key residues in the LLS structure required for its biologic activities.

Microbiota diversity

Our results put forward many crucial implications for the study of listeriosis as a disease. The status of the host microbiota has never been assessed nor taken into account in animal studies upon *L. monocytogenes* infection through the oral route. It is unlikely that *L. monocytogenes* or other enteric pathogens bet on one

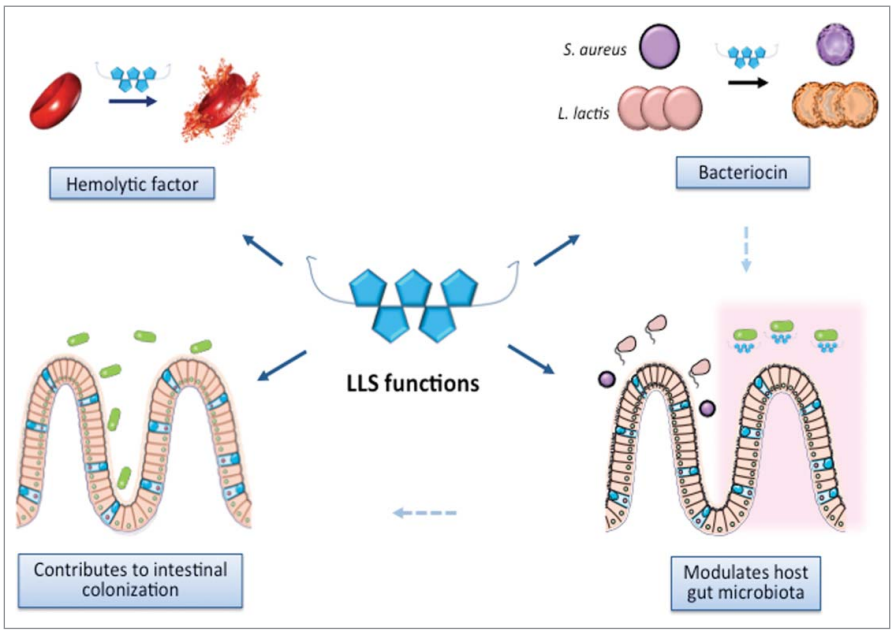

Figure 1. Functional activities of Listeriolysin S. LLS has been initially described as an hemolytic factor (top left) which contributes to the virulence of *L. monocytogenes in vivo*. Our recent work now suggests that LLS is also a bacteriocin which *in vitro* is capable of killing *S. aureus and L. lactis* (top right), and which *in vivo* modulates the host intestinal microbiota (bottom right), allowing *L. monocytogenes* colonization of the intestine (bottom left).

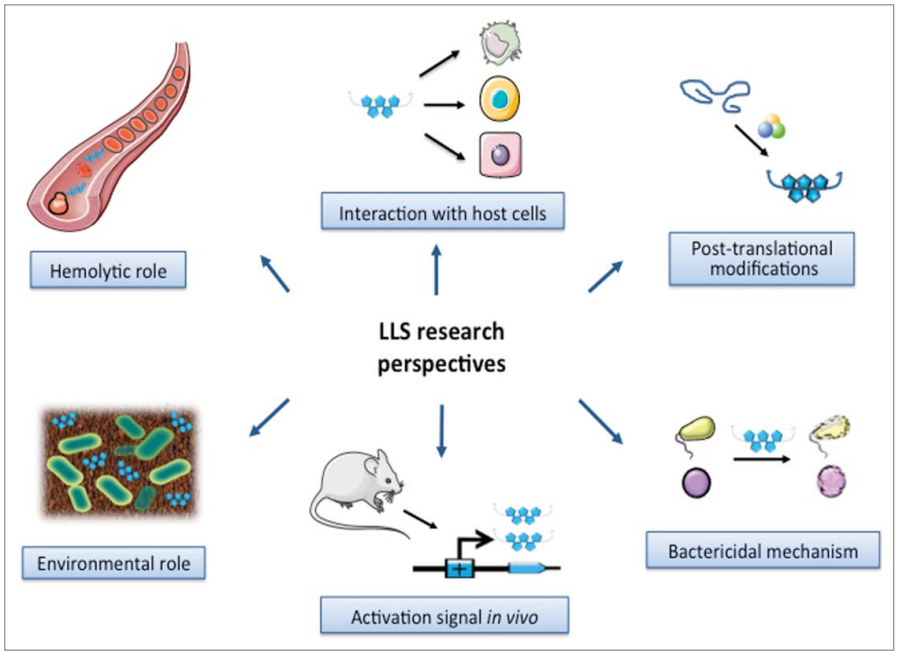

Figure 2. Perspectives on Listeriolysin (S) research. Many functions of LLS remain to be elucidated. LLS has been described as an hemolysin, but it remains to be directly demonstrated whether its hemolytic activity contributes to virulence *in vivo* (top left). Which other cellular populations may be also targeted by LLS *in vivo* has not been determined yet (top center). The structure of the post-translational modifications that characterize the mature LLS are not known (top right), and the bactericidal mechanism on susceptible species needs to be identified (bottom right). The signal that activates the expression of the LLS cluster is unknown at this stage (bottom center), and whether LLS plays a role in the survival of *L. monocytogenes* in the environment remains an open question.

single strategy to fight against intestinal microbiota: this idea makes us think that other bacteriocins or defense systems could be produced by lineage I and by lineage II strains to colonize the intestine and promote infection. We should mention also that intestinal microbiota exhibit diurnal and seasonal oscillations in composition and function.[43] Important variability observed in *in vivo L. monocytogenes* infection experiments could be explained by variations in the host microbiota of individual animals. Previous homogenization of the animal host microbiota now seems to be an important methodological requirement to compare results within animals from a single experiment. The use of controlled microbiotas in animal infection models should allow to better understand the interplay between *L. monocytogenes* and resident bacteria in the intestine.

Target species

In our recent study, we observe a significant decrease in the populations *Alloprevotella* and *Allobaculum* correlating with the production of LLS.[15] These species are producers of acetic and butyric acid, which are small molecules previously shown to either inhibit *L. monocytogenes* growth or transcriptional activity,[44,45] and therefore could be considered as 'protective microbiota species'. Whether these species are the only significant producers of acetic and butyric acid during *L. monocytogenes* infections remains to be elucidated. Interestingly, another study reported that *Allobaculum* is protective during antibiotic-induced disruption of microbiota.[46] Additionally, it is possible that *L. monocytogenes* produces other bactericions besides LLS to target other competing bacteria, expanding its capacity to control the host gut microbiota. It will be important to determine which mechanisms are involved in the reduction of *Alloprevotella* and *Allobaculum* representatives: since *Allobaculum* is a Gram-positive species (Tenericutes), it may be a direct target of LLS. *Alloprevotella* on the other hand is a Gram-negative bacterium (Bacteroidetes) and taking into account the specificity of most bacteriocins, it is highly probable that this species is not a direct target of LLS: the decrease in *Alloprevotella* would therefore be indirect, as a result in the decrease of another species that probably controls *Alloprevotella* growth. Identifying the different interactions between

microbiota species during lineage I *L. monocytogenes* infection will be crucial to understand the contribution of LLS to infection.

Therapeutic uses

We still do not know which bacterial species are controlled by LLS in humans, but our results and data from other teams highlight that protective bacterial species could be used as a strategical treatment to prevent *L. monocytogenes* infection in individuals at risk. The group of Colin Hill and colleagues has elegantly shown that bacteriocin production by *Lactobacillus salivarius* UCC118 allows mice protection against oral *L. monocytogenes* infection.[47] Moreover, they have also shown that thuricin CD, a 2-peptide bacteriocin from *Bacillus thuringiensis*, was found to display antimicrobial activity comparable to the activities of vancomycin and metronidazole in a model of human distal colon infection by *Clostridium difficile*, without changing dramatically the composition of the commensal microbiota (which is observed during vancomycin and metronidazole treatment).[48] In the same line, engineering of LLS may potentially be used to treat infection caused by microorganisms such as *S. aureus* which is already resistant to multiple antibiotics.

Activation signal

We tried to identify specific signals which trigger *in vitro* the expression of LLS, including mucin, gastric fluid, trypsin, pepsin, NaHCO3, bile salts, detergents, succinic acid, propionic acid, valeric acid, ethanolamine, antibiotics, 6% 0_2 or intestinal content added *ex vivo*. However, no signal is able to activate the promoter of the LLS operon.[15] As expression of several bacteriocins is triggered precisely by the presence of other bacteria,[49] we hypothesized that the intestinal microbiota could be precisely the signal recognized by *L. monocytogenes* to induce LLS expression. Nevertheless, experiments in germ-free mice indicate that the LLS promoter may be activated in the absence of microbiota.[15] These results suggest that LLS is probably produced upon exposure to a pleiotropic set of conditions that may combine several parameters. Detection of the LLS activation signals is therefore an important research topic, not only during host infections but also in environmental conditions (see below).

Environmental role

It has been observed that the LLS cluster is present in several isolates of the non-pathogenic species *Listeria innocua*: in some of them, the cluster presents many mutations and it is not functional but in others, the produced LLS is fully hemolytic.[50] The significance of these results is not clear, but may reveal a role for LLS in the environment. Several bacteriocins are produced for niche colonization, and it would not be surprising to discover such a role for *L. innocua* and also for *L. monocytogenes* in the environment. These results also call into attention the evolution of other members of the LLS biosynthetic cluster in *Clostridium botulinum, S. pyogenes* and *S. aureus*, all of them pathogens with different lifestyles. How the regulation and function of this family of TOMM evolved in these bacterial pathogen remains an attractive field for future investigations.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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