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Bacterial and cellular RNAs at work during *Listeria* infection**Abstract/Summary**

Listeria monocytogenes an intracellular pathogen that can enter and invade host cells. In the course of the infection, RNA-mediated regulatory mechanisms provide a fast and versatile response for both the bacterium and the host. They regulate a variety of processes such as environment sensing, and virulence in pathogenic bacteria as well as development, cellular differentiation, metabolism and immune response in eukaryotic cells. The aim of this review is to summarize first the RNA-mediated regulatory mechanisms playing a role in the *Listeria* lifestyle and in virulence and then the host miRNA response to *Listeria* infection. Finally, we discuss the potential crosstalk between bacterial RNAs and host RNA regulatory mechanisms as new mechanisms of bacterial virulence.

Keywords

virulence, sRNA, asRNA, riboswitch, thermosensor, excludon, CRISPR, miRNA, immune response, RNA secretion

Introduction

The human pathogen *Listeria monocytogenes* ranks among the best-known invasive bacteria. In the course of the infection of susceptible individuals, primarily elderly and pregnant women, *Listeria* can cross the intestinal, blood-brain and fetal-placental barriers causing a disease known as listeriosis. *Listeria* is an intracellular pathogen that has the ability to invade, survive and actively multiply within professional phagocytes and a number of non-phagocytic cells. During infection, *Listeria* produces a plethora of virulence factors whose production is spatio-temporally regulated by both protein-mediated and RNA-mediated regulatory mechanisms. The secreted and surface exposed virulence factors allow *Listeria* to deploy a number of sophisticated strategies to compromise the cell and also promote its survival. These involve adherence and entry into the mammalian cells by exploiting host cell receptors and signalling events, manipulation of the immune defence mechanisms, impairment of organelle dynamics and interference with post-translational modifications. Recent studies have highlighted that *Listeria* could also reprogram the host cell transcription by inducing histone modifications, chromatin remodelling and by impacting on the miRNA expression profiles of infected cells and tissues [1-4].

The mechanisms underlying mammalian and bacterial gene regulation share remarkable similarities. Besides protein regulators, non-coding RNAs (ncRNAs) are increasingly recognized as highly versatile regulatory components in both eukaryotes and prokaryotes. Their roles range from transcription regulation to translation repression and chromatin remodelling. Prokaryotic ncRNAs have important roles in mediating the response to environmental cues, in performing housekeeping functions and in controlling the virulence in pathogenic bacteria [5, 6]. The first ncRNAs in *Listeria* were identified by co-immunoprecipitation with Hfq, a small RNA-binding protein

required for small RNAs function in bacteria [7] and by an *in-silico* based approach [8]. However, major progress in the discovery of regulatory RNA transcripts were made with the use of high-density tiling arrays and RNA-Seq [9-13], which provided a picture of the whole *Listeria* transcriptome in multiple conditions. This led to the annotation of hundreds of regulatory RNAs in *Listeria* among which some play regulatory roles in virulence [14]. Likewise, eukaryotic ncRNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), regulate a variety of processes such as development, cellular differentiation, metabolism, immune response as well as viral and parasite infections [15-18]. More than 1000 miRNAs are annotated in the human genome and it is predicted they could regulate 60% of the human transcriptome [19].

The aim of this review is to highlight the importance of RNA-mediated regulatory mechanisms, both in *Listeria* and in the infected mammalian cell, which play a role in the subtle pathogen-host interactions, dictating the progress of the infection. We will first review the known RNA-mediated regulatory mechanisms controlling the *Listeria* virulence and then our current knowledge on the expression of eukaryotic miRNAs in the response to *Listeria* infection. Finally, we will speculate on the potential crosstalk between bacterial and host RNA regulatory mechanisms during the infection.

I. The *Listeria* regulatory RNA repertoire important for the virulence process

Bacterial regulatory RNAs can be classified into several groups: 5'-untranslated regions (5'-UTRs) of mRNAs, *cis*-encoded antisense RNAs (asRNAs), *trans*-acting small RNAs (sRNAs) and the more recently described, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs). In the following section, we will briefly describe the main regulatory principles characteristic for each class, and further detail the specific examples of molecular mechanisms found to have an impact on *Listeria* virulence.

The 5'-untranslated region (5'UTR) of an mRNA is located between the transcriptional start site (TSS) and the translational initiation site. It harbours the Shine-Dalgarno (SD) sequence to which the ribosome binds and initiates protein translation. In prokaryotes, transcription and translation are coupled and therefore, many 5'-UTRs have evolved as efficient gene expression regulators that sense physicochemical signals (e.g. thermosensors and riboswitches), or can bind proteins and RNA regulators acting before completion of the transcription/translation of the gene. The precise length of all 5'-UTRs in the *Listeria* transcriptome has been recently determined by high resolution mapping of the TSSs in a genome-wide manner [13]. A group of 101 genes with an unusually long 5'-UTR (>100nt) includes 10 known *Listeria* virulence factors [13] among which some have been extensively studied.

The main regulator that orchestrates the *Listeria* infectious process is PrfA (Positive regulatory factor A), a transcription factor of the Crp/Fnr family that induces the expression of major known virulence genes. Its expression is tightly regulated by two RNA-mediated mechanisms operating at its 116 nucleotide long 5'-UTR (Figure 1). First, the 5'-UTR of *prfA* mRNA is a **thermosensor** element, which adopts a stable stem-loop structure at a low temperature, thereby occluding the SD sequence and preventing binding of the ribosome. When the temperature increases to 37°C, the stem-loop melts into an alternative secondary structure, allowing the ribosome to access the SD sequence, leading to the translation of the *prfA* mRNA and to the subsequent induction of a number of virulence genes [20]. A second mechanism of *prfA* expression regulation involves a **trans-acting riboswitch-derived** element. Typically, riboswitches are 5'-UTR elements that, upon binding of a ligand (tRNA, ions or metabolites), undergo conformational changes and affect the transcription or the translation of a nascent mRNA transcript. Riboswitch-regulated transcripts usually encode genes involved in the

94 biosynthesis of the molecule that regulates the riboswitch[21]. In the case of *Listeria*, the
95 short transcript of the SAM(S-adenosyl-methionine)riboswitchSreA, which regulates in
96 *cis* the expression of genes involved in methionine and cysteine metabolism, interacts in
97 *trans* with the 5'UTR of *prfA*mRNA, approximately 80 bases upstream of the SD site.This
98 binding decreases the translation of *prfA*[22]. This is the first, and so far unique example
99 of such a dual function for a riboswitch element. The PrfAthermosensor-mediated
100 temperature sensingand the riboswitch-mediated nutrient sensing allow *Listeria* to
101 sense its environment and accordingly regulate PrfA expression, turning on
102 theexpression of crucial virulence genes solely when required in the host.

103 **Cis-encoded antisense RNAs (asRNAs)**are heterogeneous groups of regulatory
104 transcripts that originate from the DNA strand opposite to genes they regulate, or can
105 arise from overlapping 5'UTRs and 3'UTRs of adjacent genes. In all cases, *cis*-encoded
106 antisense transcripts have perfect complementarity with the sense transcript and are
107 denoted as antisense RNAs (asRNAs). Their length varies dramatically, ranging from less
108 than a hundred to several thousand nucleotides, overlapping one or several genes. In
109 *Listeria* there are 95asRNA transcripts annotated to date, whose function is in most
110 cases unknown.

111 Of note, for some of the long asRNA transcripts, a recurring pattern was observed
112 in at least 13 characteristic antisense containing genomic loci, which led to the definition
113 of a novel concept in bacterial gene regulation named **excludon**[13, 23](Figure 2A). The
114 excludon is a locus encoding two divergent genes with related and often opposite
115 function and a long asRNAof one gene, thatalso contains the mRNA of the
116 divergentadjacentgene. In two cases, it was demonstrated thatthe asRNA negatively
117 affects the expression of the overlapped gene whereas its distal part constitutes a
118 functional mRNA and positively contributes to the expression of the adjacent gene[12,

13]. In other words, an excludon functions as a genomic toggle where a single transcript governs the mutually exclusive expression of adjacent genes that generally have opposing functions. For example, an excludon regulates the transcription of flagellar/motility genes [12] (Figure 2A). Flagella are important mediators of *Listeria* pathogenicity [24] but at the same time, they are strong inducers of the host immune response [25] and therefore, their tight regulation is crucial for *Listeria* survival during infection.

The diversity of asRNA-mediated regulation is further illustrated by the remarkable example of a **riboswitch-regulated asRNA** in *Listeria* [26] (Figure 2B). A vitamin B12-dependent riboswitch regulates the expression of the asRNA *AspocR*, which overlaps the gene encoding *PocR*, a transcription factor that activates transcription of the genes mediating propanediol catabolism (*pdu*) and vitamin B12 biosynthesis (*cob*). Vitamin B12 is an important cofactor for the activity of diol-dehydratase, an enzyme required for propanediol catabolism. In the presence of B12, the riboswitch terminates prematurely *AspocR* transcription, allowing the subsequent expression of *pocR*, whereas in the absence of B12, *AspocR* is fully transcribed, thus negatively regulating *PocR* production. Interestingly, the negative regulation of *pocR* expression was observed when *AspocR* was expressed *in trans*, indicating that it likely interferes with the transcription or translation initiation of *pocR*. Overall, this mechanism ensures that *PocR* is produced uniquely when the B12 cofactor is available, allowing the subsequent activation of the propanediol catabolism genes. Propanediol, together with the closely related metabolite, ethanolamine, constitute important nutrient sources for bacterial enteropathogens [27]. Recently, it was shown that during intestinal infection by *Salmonella enterica*, use of ethanolamine as a carbon source enables the bacterium to outcompete the intestinal microbiota that cannot use this nutrient [28]. Accordingly, the

expression of genes involved in the utilization of propanediol and ethanolamine are up-regulated during intracellular growth of *Listeria* [29] and more interestingly, also in *Listeria* isolated from the intestine of germ-free mice pretreated with lactobacilli [30], suggesting their important role in *Listeria* virulence.

Trans-encoded small RNAs (sRNAs) are transcribed from intergenic regions, or are generated by processing of the 5'UTRs or 3'UTRs of mRNAs, and in contrast to the *cis*-sRNAs, they regulate targets encoded at distant genetic loci. The most extensively studied *trans*-encoded sRNAs are those targeting mRNA molecules. They can also bind and sequester proteins. The interaction between a sRNA and its target mRNA is mediated by short, imperfect base pairing and can either positively or negatively affect the target transcript [6]. In *Listeria* there are more than 150 transcripts annotated as sRNAs and similarly to the asRNA transcripts, their biological function is in most cases unknown [7-13]. However, important information about their expression conditions, and hints into their potential function, was obtained by extensive tiling array analysis using bacteria grown in four physiologically relevant conditions (exponential phase, stationary phase, hypoxia and low temperature), or isolated from intestine of axenic mice or bacteria grown in blood of human donors. The same panel of conditions was used to analyse mutants of known virulence regulators and RNA binding proteins ($\Delta prfA$, $\Delta sigB$, Δhfq) [12, 13]. Likewise, RNA sequencing with the 454 technology of *Listeria* grown in macrophages, revealed sRNAs whose expression is induced during the intracellular phase of the infection [10]. Assuming that sRNAs are generally induced in conditions relevant for their biological role, these studies highlighted sRNAs whose function might be important for *Listeria* virulence, and enabled the prediction of their potential regulators. In addition, a number of sRNAs annotated in the *L. monocytogenes* genome are not conserved in the closely related, but non-pathogenic

species *L. innocua*[13]. Comparative genomic studies of the two species have been previously used to identify a number of *Listeria* virulence factors[1, 31], and it is thus tempting to speculate that *L. monocytogenes*-specific sRNAs would play a role in virulence. Indeed, nearly all sRNAs shown to have a role in virulence are absent from thenon-pathogenic species. Among these, Rli33-2 and Rli50, when deleted, led to an attenuated virulence phenotype in murine macrophage infection as well as in mouse and butterfly larvae infection models[10]; similarly, a deletion mutant of Rli38 resulted in an attenuated virulence phenotype in orally inoculated mice [12]. Another sRNA absent from *L. innocua*Rli27has been recently shown to positively regulate the expression of *lmo0514*, encoding an LPXTG surface protein enriched in the cell wall of intracellular bacteria[32]. This regulation occurs by mechanism involving pairing of Rli27 with the 5'UTR of the *lmo0514* mRNA. Remarkably, *lmo0514* transcript is detected in two variants, differing in length and in relative amount in extra- and intracellular bacteria. Only the long version, more abundant in intracellular bacteria, contains the 5'-UTR recognized by the Rli27,rendering this regulation possible only inside the host cell (Quereda, et al.PLoS Genetics, *in revision*). Some sRNAs might have multiple target genes, as shown in the case of LhrA which affects expression of nearly 300 genes and directly regulates expression of *lmo0850*, *lmo0302* encoding proteins with an unknown function and *chiA*encoding a chitinase[33, 34]. ChiA contributes to *Listeria* pathogenesis [35].

It is worth mentioning that some *Listeria* sRNAs annotated as non-coding transcripts encode putative open reading frames (ORFs) for small, often very basic polypeptides, whose function is unknown. As reported for other species, these peptides could act as signaling molecules involved in bacterial communication or might play a role in bacterial virulence [36-38].

CRISPR/Cas systems (Clustered Regularly Interspaced Short Palindromic Repeats) provide bacteria and archaea with specific mechanisms of RNA-mediated adaptive immunity against invading nucleic acids, i.e. viruses and conjugative plasmids. Typically, CRISPR systems are composed of arrays of identical repeat sequences, interspaced with non-repetitive variable spacers, coupled with clusters of CRISPR-associated (*cas*) genes that are involved in all steps of CRISPR function. At the core of CRISPR functionality are the spacers, short DNA segments originating from a foreign DNA, which when transcribed provide a specific guide for CRISPR-mediated DNA/RNA silencing of the corresponding invading virus or a plasmid[39]. *Listeria* species encode three different CRISPR systems[40-42]. CRISPR-I and/or CRISPR-II are present in some *Listeria* strains and are always associated with *cas* genes. Their identified spacers match uniquely *Listeria* bacteriophages. The third CRISPR, the RliB-CRISPR (previously annotated as a sRNA named RliB) is present in all so far sequenced *Listeria* strains but is never associated with a *cas* locus. However, both in the *cas*-less *Listeria* strains and in those encoding a complete set of *cas* genes elsewhere in the genome (adjacent either to CRISPR-I or CRISPR-II), the RliB-CRISPR is expressed and processed [42]. Surprisingly, this processing is governed by the polynucleotide phosphorylase (PNPase), a genome-encoded bi-functional enzyme harboring both 3' to 5' exonuclease and 3' polymerase activities[43]. The identification of RliB-CRISPR processing by PNPase revealed a unique role for this enzyme in bacterial "CRISPRology". Similarly to CRISPR-I and CRISPR-II, RliB-CRISPR targets *Listeria* bacteriophages. Functional studies of RliB-CRISPR showed it has a DNA-interference activity. Singularly, its activity requires that both PNPase and the *cas* genes belonging to CRISPR-I are present in the genome. RliB-CRISPR and CRISPR-I share a similar repeat sequence, suggesting they might share the same enzymatic machinery required for their function[42]. Interestingly, RliB-CRISPR is

conserved in pathogenic *Listeria* species and its expression is significantly up-regulated in bacteria isolated from the intestinal lumen of gnotobiotic mice and in bacteria grown in the human blood. The *L. monocytogenes* EGD-emutant deleted for RliB-CRISPR colonized liver of intravenously inoculated mice better than the wild type bacteria [12]. This phenotype was however opposite when mice were inoculated by the oral route (our unpublished data), suggesting that RliB-CRISPR might be important during the intestinal phase of the infection. Indeed, the human gut microbiome is rich in bacteriophages and CRISPR systems are highly dynamic in such an environment [44, 45]. Therefore, RliB-CRISPR contribution to *Listeria* virulence might be indirect by impacting the bacterial survival challenged by bacteriophages. Additionally, during *Listeria* intracellular infection, a temperate prophage is excised, reconstituting a functional *comK* gene which promotes bacterial escape from the phagosome [46]. Whether RliB-CRISPR, CRISPR-I or CRISPR-II contribute to the control of the prophage excision, remains to be examined. Altogether, RliB-CRISPR reveals the importance of the interactions between bacteriophages and bacteria during saprophytic life and during infection.

As a result of high throughput transcriptome studies a comprehensive overview of the *Listeria* non-coding genome in multiple growth conditions relevant for the infectious process is publicly available (http://www.weizmann.ac.il/molgen/Sorek/listeria_browser/). The functional studies have revealed a broad diversity of regulatory mechanisms underlying the action of individual RNAs. A future challenge will be to decipher the biological function of the many annotated, but so far unexplored ncRNAs in *Listeria*. Altogether, recent research on *Listeria* RNA-mediated regulations, as well as the impressive number of studies in other

bacterial pathogens[47, 48], clearly points toncRNAs as crucial contributors to the virulence process.

II. The mammalian miRNA response to *Listeria* infection

MicroRNAs (miRNAs) are 21-24 nucleotide long regulatory RNAs present in animals, plants and viruses. They are derived from a long primary transcript (pri-miRNA) that is first processed in the nucleus by the RNaseIII family dsRNA-endonuclease Drosha into a pre-miRNA. The pre-miRNA is exported into the cytoplasm and further cleaved by another member of the RNase III family, Dicer. Processed single stranded miRNAs associate with the RNA-induced silencing complex (RISC), consisting of multiple proteins among which members of the argonaute protein family have RNase activities, and are central to the RISC function [49]. The miRNA interaction with the target mRNA is mediated by imperfect complementarity between the 3'-UTR of the target transcript and the miRNA-RNase ribonucleoprotein complex and it typically leads to translation inhibition and/or degradation of the target gene. To achieve effective processing, this interaction requires a so called "seed region", a sequence harboring perfect complementarity with the 5'-end of the miRNA [50].

As previously mentioned, miRNAs are involved in various physiological and pathological processes. Their role during bacterial infections of animals has only recently started to be investigated with several pioneering studies, e.g. in *Helicobacter pylori*, *Salmonella enterica* and *Mycobacterium avium*[51-54]. The role of miRNAs during *L. monocytogenes* infection has been addressed both in cultured cells[55, 56] as well as *in vivo* in mice models[30, 54, 57]. Here, those studies will be presented in an order, which may look awkward but follows the course of the natural infectious process.

Listeria infection starts by ingestion of contaminated food, which delivers the bacterium to the **intestinal lumen** of the host. There, *Listeria* competes with the intestinal microbiota in order to colonize the lumen, cross the intestinal barrier and further disseminate to deeper organs. A study examining the impact of lactobacillion orally acquired listeriosis [30] and a study addressing the role of microbiota in the regulation of miRNA expression in the ileum of *Listeria* infected mice [57] identified a particular expression response of protein-coding genes and interestingly, of miRNA regulators (Figure 3). These two comprehensive studies represent the first *in vivo* evidence of a particular miRNA signature induced during orally acquired *Listeria* infection. More interestingly, expression of several infection-induced miRNAs, such as miR-192, miR-143, miR-148a, miR-200b and miR-200c was affected by the presence of lactobacilli or the host microbiota, demonstrating the important role of intestinal bacteria in the modulation of the host miRNA response to infection [30, 57]. A single miRNA family was common to both studies, i.e. miR-200, which has been reported to induce epithelial differentiation and suppress the epithelial-mesenchymal transition in several types of cancer [58] as well as to play a significant role during the *Helicobacter* infection [59]. The miRNA target prediction results crossed with the transcriptomic data revealed that miR-200 and other regulated miRNAs could target genes with a function in immunity as well as genes whose function could be related to the infection. Some miRNAs could target the same protein-coding genes, suggesting the existence of complex miRNA-mRNA regulatory networks [30, 57]. Importantly, expression of some of the predicted targets anti-correlated with the expression of the putative miRNA regulator during the *Listeria* infection, e.g. an immune response transcription factor (*Atf3*), a retinoic acid induced protein that plays a role in epithelial cell differentiation (*Gprc5*), an enzyme involved in fucosylation of epithelial cells (*Fut2*), a protein that plays

a role in intestinal inflammation (*Nt5e*) and an RNA editing enzyme of the miRNA and small interfering RNA (siRNA) pathways (*Adar*), supporting that predicted interactions indeed might occur in the infected tissue. Moreover, a number of interactions were predicted to occur both in mice and humans. Their conservation in significantly distant organisms furthermore supports the validity of their biological function.

Following infection, *Listeria* needs to overcome the rapidly triggered host innate immune response. Early resistance to the *Listeria* infection relies in part on the production of interferon- γ (IFN- γ) by **natural killer (NK) cells**, which promote the activation of macrophages [4]. Ma et al. reported that IFN- γ expression is regulated by miR-29, which directly binds within the 3'UTR of the *ifn- γ* mRNA. Interestingly, mice infected with *Listeria* showed decreased expression of miR-29 and a relevant increase in the production of IFN- γ . Moreover, transgenic mice expressing a sponge target construct that competes with endogenous miR-29 targets, displayed a lower bacterial burden in comparison to the wild type mice, indicating that lower expression of miR-29 and higher IFN- γ production in NK cells, promoted host resistance to *Listeria* infection [56].

In the following steps of the infection, *Listeria* is internalized by **macrophages**. During the infection of bone marrow derived macrophages (BMDMs), *Listeria* induces expression of 13 miRNAs among which miR-155, miR-146a, miR-125a-3p/5p and miR-149 are the most significantly up-regulated [54]. This induction occurs already when bacteria are in the phagosome and is mediated by MyD88, a universal adaptor protein used by almost all Toll-like receptors (TLRs) to activate the transcription factor NF- κ B, a key regulator of the immune response to the infection. Indeed, miR-155 and miR-146 are known modulators of the immune response in macrophages [60, 61], whereas the functions of miR-125a-3p, miR-125a-5p and miR-149 have not yet been described. Target prediction analysis suggested that all 5 miRNAs could potentially interact with

mRNAs encoding immune-related proteins. For instance, miR-125a-3p and miR-125a-5p could respectively target the interleukin-1 receptor 1 (*Il-1R1*) and IL-6 receptor (*Il-6 R*) transcripts[54].

The whole infectious process relies on the *Listeria* capacity to enter non-phagocytic cells. During infection of **epithelial** cells, *Listeria* induces expression of miR-155, miR-146b and miR-16 and decreases expression of let-7a1 and miR-145, all of which are also implicated in the regulation of immune-related genes. Interestingly, several major *Listeria* virulence determinants, the surface internalins InlA and InlB as well as the secreted toxin listeriolysin O (LLO), are implicated in the regulation of the above-mentioned miRNAs [55]. Purified LLO could fully reproduce the *Listeria*-induced miRNA expression profile whereas a *Listeria* deletion mutant for *inlA* and *inlB* led to decreased expression of miR-155, suggesting a putative role for internalins or *Listeria* entry in miRNA regulation [55].

After a primary infection, *Listeria* stimulates a strong memory **CD8⁺ T-cells** response, allowing a rapid clearance of the bacteria from the infected tissues upon a re-infection [62]. Interestingly, in knock-out mice not expressing miR-155, the CD8⁺ T-cell response is significantly reduced following *Listeria* infection, indicating that this miRNA has an important role in the regulation of the CD8⁺-mediated response to the infection by an intracellular pathogen [63]. However, the direct effect of *Listeria* on the expression of the miR-155 in this cell type is not known.

A significant effort has been made to identify numerous mammalian miRNAs, both *in vivo* and in different cellular models, whose expression is regulated during *Listeria* infection. Not surprisingly, the miRNA profile induced in the intestinal tissue is different from that induced by a *Listeria* infection in different cell lines. Nevertheless, the regulated miRNAs share similar functions (either predicted or experimentally described),

mainly regulating immune genes. Indeed, miRNAs are key components of the innate immune response [15, 64] and previously mentioned studies suggest that miRNAs are crucial regulators of host defenses against intracellular bacterial infection, but also potential targets for the pathogen-induced manipulation and/or evasion of the host immune response. Similarly to the miR-200 family, which is specific to the intestinal miRNA response, miR-155 and miR-146 appear to be induced by *Listeria* in different cellular contexts – BMDMs and epithelial cells. Interestingly, these miRNAs are also induced by other bacterial pathogens, e.g. *Helicobacter pylori* [51, 65], *Salmonella enterica* [52], *Mycobacterium avium* [53] as well as viral and fungal pathogens [66, 67], indicating their universal role in the common immunity pathways shared by different pathogens. In line with this remark, the expression of miR-155 and miR-146 is controlled by NF- κ B pathway, which regulates a number of genes critical to innate and adaptive immunity, cell proliferation, inflammation, and tumor development [64].

Although identification of the miRNA profile during *Listeria* infection is clearly underway, a future challenge will be to decipher the molecular mechanism underlying the miRNA expression changes upon infection as well as to identify their relevance for the *Listeria* infectious process.

III. A potential crosstalk of bacterial and mammalian regulatory RNAs during *Listeria* infection

As emphasized in the introduction, *Listeria* has evolved a number of sophisticated strategies to establish an efficient infection and promote its survival in the host. The *Listeria* effectors known to be involved in these complex roles include LLO, which forms pores, promotes escape from the vacuole, triggers histone modifications, other post-translational modifications and mitochondrial fragmentation, ActA which allows

Listeria to move intracellularly, InlC that interferes with NF- κ B activation and LntA, which enters the host nucleus and induces chromatin remodeling. All these virulence factors are all proteinaceous molecules[1]. It is tempting to speculate that numerous *Listeria* ncRNAs for which the functions have not been identified, might as well act as such effectors i.e. RNA virulence factors that could be actively delivered to the host cell and manipulate host regulatory pathways.

While such RNA effectors have never been described in bacterial pathogens, and while it was never formally demonstrated that a specific bacterial RNA is actively delivered to the host cell, there is a strong logic supporting the existence of bacteria-host RNA-mediated communication. First, many pathogenic bacteria as exemplified by *Listeria*, enter the host cell and therefore have access to different cellular compartments. Second, they possess various systems of export/secretion that can secrete proteins, and also nucleic acids. For instance, it has been shown that *Neisseria gonorrhoeae* can secrete single stranded DNA by the type IV secretion system (T4SS) [68]. In the case of *Listeria*, it has been shown that it can secrete small nucleotides such as c-diAMP [69], and it was recently demonstrated it can also release DNA and RNA during the infection of the host cell [70]. Third, regulatory RNAs offer a general mechanism to interfere with mammalian regulation. For example, a bacterial RNA could bind a host miRNA and inhibit its function or it could mimic a mammalian miRNA, thereby overtaking the miRNA-machinery and affecting the expression of the host genes. As emphasized in the preceding section, miRNAs are key components of the immune response [15, 64], which makes them exceptional targets for such pathogen-induced manipulation. As shown with *Listeria* and other pathogens, their expression is indeed significantly affected upon infection. Alternatively, one should not exclude the possibility that RNA virulence

effectors might affect function of other host ncRNAs, such as lncRNAs, or could bind and sequester host regulatory proteins as well as have other, yet unanticipated functions.

A direct evidence of such host-pathogen cross-kingdom RNA-mediated regulation comes from some remarkable studies of viral and fungal pathogens. *Herpesvirus saimiri* (HVS) and also cytomegalovirus (CMV) express RNAs that interact and lead to degradation of the host miR-27, consequently affecting the expression of miR-27 target genes [71-73]. A fungal pathogen *Botrytis cinerea* expresses a set of sRNAs, which mimic host miRNA and bind to Argonaute 1 protein (AGO1), selectively silencing a subset of host immunity genes [74].

It has been recently shown that during infection, viable *Listeria* can release nucleic acids in the host cytoplasm [70]. As said above, this occurs also for other pathogenic bacteria and is essential for generation of anti-microbial immunity [75]. Cytosolic *Listeria* can release the second messenger c-di-AMP [69] as well as RNA/DNA [70, 76] that are recognized by the sensors RIG-I, MDA-5 and STING, resulting in the production of IFN. Bacterial RNAs are exceptionally good PAMPs (Pathogen Associated Molecular Patterns – molecules associated with pathogens that are recognized by the innate immunity) as they differ from the eukaryotic RNA by the nature of their 5'-end, which instead of a trimethylguanosine cap, consists of a triphosphate. RIG-I has been shown to recognize triphosphorylated *Listeria* RNA [76]. More importantly, translocation of RNA during the *Listeria* infection was visualized in the host cytosol using a sensitive RNA fluorescence technique [76] and this translocation was shown to be dependent on the activity of SecA-2 [70], an auxiliary protein secretion system that promotes secretion of several virulence factors [77] as well as other genes whose expression is strongly induced *in vivo* [78]. These data strongly indicate that the translocation of nucleic acids

during the infection is not a product of bacterial lysis but might be governed by an active bacterial process.

Even though these studies provide evidence that *Listeria* RNA has an access and is actively delivered to the host cytosol during the infection, nothing is known about the specificity of this process and its potential benefit for the pathogen. A secreted RNA virulence factor has never been identified in bacteria and this exciting hypothesis remains to be explored in the future.

IV. Conclusions

Decades of research led to the discovery of numerous *Listeria* molecular strategies, which have been selected during the billions of years of pathogen-host coevolution, to establish a successful infection. ncRNAs are versatile regulators important for the *Listeria* virulence gene expression, metabolism regulation and the interaction with the host. Similarly, eukaryotic miRNAs are potent regulators controlling the expression of the human genome with an important accent on the immune response regulation. This makes them a potent target for pathogen manipulation. Indeed, during different phases of the *Listeria* infectious process, the host miRNA expression is significantly altered. Similarity between prokaryotic and eukaryotic RNA-mediated molecular mechanisms and the accessibility of the host RNA machinery to the intracellular *Listeria* highlights a possibility of the interspecies RNA crosstalk between the pathogen and the host.

V. Future perspectives

As revealed by transcriptomic studies, most of the *Listeria* genome is expressed, however little is known about the biological function of many transcripts. Exploration of

their function will certainly reveal new principles of gene regulation in bacteria. Our understanding of *Listeria* interaction with the mammalian miRNA regulatory pathways is still in its infancy. Most of the studies performed so far are descriptive, yet they achieved significant progress in recording miRNA expression changes in the host upon *Listeria* infection. A future challenge will consist in deciphering(a) how *Listeria* targets a specific set of miRNAs during a particular phase of the infectious process, (b) what are the regulated target genes, and (c) what is the direct benefit for the bacterium as well as for its virulence.

Up to now, the known *Listeria* virulence effectors are protein molecules. Being aware of the versatile nature and immense regulatory capacity represented by RNA molecules, and supported by the studies in viral and fungal pathogens, one can imagine the exciting hypothesis that such secreted virulence effectors might also be RNAs. The identification of such RNA effectors would open new horizons in the studies of pathogen-host interactions and the field of cellular microbiology.

Executive summary:

Listeria regulatory RNA repertoire important for the virulence process

- *Listeria monocytogenes* is an invasive pathogenic bacterium whose virulence factors expression is controlled by RNA-mediated regulatory mechanisms.
- The expression of the main *Listeria* virulence regulator PrfA is regulated by an RNA thermosensor and a trans-acting SAM riboswitch.
- In *Listeria*, 13 long asRNAs named excludon, which regulate expression of genes with opposite functions and act as fine-tuning regulatory switches, have been identified.
- The *Listeria* vitamin B12 biosynthesis and propanediol catabolism, an important nutrient during the intestinal phase of the infection, is controlled by the transcription factor PocR. Expression of PocR is regulated by the B12 riboswitch-regulated asRNA AsPocR.
- There are more than 150 annotated sRNAs in *Listeria* with mostly unknown functions. For some of them it has been shown a role in virulence.
- *Listeria* RliB-CRISPR system, which is processed with the help of chromosomally encoded polynucleotide phosphorylase (PNPase), has a role in the virulence process.

The mammalian miRNA response to *Listeria* infection

- During the infection, *Listeria* induces expression changes of the host miRNAs, mainly regulating immune genes.
- The miR-200 family is specific to the intestinal miRNA response after orally induced listeriosis and miR-155 and miR-146 appear to be induced by *Listeria* in different cellular contexts.

A potential crosstalk of bacterial and mammalian regulatory RNAs during *Listeria* infection

- Viable *Listeria* can release nucleic acids in the host cytoplasm which might have a regulatory function to favor the infection.

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Shows *Listeria* nucleic acids are released during the host cell infection via SecA2 dependent pathway.

Figures:

Figure 1. 5'UTR-mediated regulation of PrfA expression.

At temperatures below 37°C, the 5'UTR of *prfA*mRNA forms a stable hairpin structure that occludes the Shine-Dalgarno sequence (SD) and prevents binding of the ribosome. At 37°C this structure melts, allowing the ribosome to bind and produce the PrfA protein that activates expression of many virulence genes. In addition, at 37°C the transcript generated by the S-adenosyl-methionine (SAM) riboswitch (SreA) interacts with the *prfA* 5'-UTR and prevents the production of the PrfA protein.

Figure 2. The asRNA-mediated mechanisms of gene regulation in *Listeria*

A) Example of an excludon, where a long asRNA Anti0677 overlaps and serves as an antisense regulator of *Imo0675*, *Imo0676* and *Imo0677* encoding FliN, FliP and FliQ, respectively, which are components of the flagellum export apparatus, while simultaneously encompassing the 5'-UTR and the mRNA of *Imo0674* encoding MogR, a transcriptional repressor of the flagellum genes. The expression of Anti0677 is regulated by sigmaB (σ_B , a stress-activated transcriptional regulator). Altogether, the excludon ensures that by two mechanisms (inhibition mediated by the antisense component of anti0677; and repression mediated by increased expression of the MogR repressor)

flagellum production is switched off. **B)** The vitamin B12-dependent riboswitch regulates expression of the asRNA *AspocR*, which overlaps the gene encoding *PocR*. In the absence of vitamin B12, the riboswitch forms an anti-terminator structure, which allows the transcription of *AspocR*, resulting in the decreased production of the *PocR*. In the presence of vitamin B12, the riboswitch generates a short transcript, allowing increased production of *PocR* transcription factor.

Figure 3. Regulation of the host miRNA expression during *Listeria* infection.

Schematic representation of the significantly regulated miRNAs in the intestinal tissue during orally acquired listeriosis (grey) and in infected cell lines (blue, purple, orange and green). Highlighted are the miRNAs whose expression is modulated by the presence of the host microbiota or lactobacilli: expression decrease upon *L. monocytogenes* infection and expression increase upon treatment with *Lactobacillus* (*), expression decrease only in the presence of microbiota upon *L. monocytogenes* infection (**), expression decrease in the presence of microbiota and expression increase in the absence of microbiota mice upon *L. monocytogenes* infection (***). In bold are miRNAs detected to vary during different infections.

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Type of RNA regulator	Specific name	Study (year)	Ref.
5'-UTR			
Thermosensor	5'-UTR <i>prfA</i> mRNA	Johansson <i>et al.</i> (2002)	[20]
<i>Trans</i> -acting riboswitch	SAM SreA	Loh <i>et al.</i> (2009)	[21]
<i>Cis</i>-encoded asRNAs			
Excludon	Anti0677	Toledo-Arana <i>et al.</i> (2009), Wurtzel <i>et al.</i> (2012), Sesto <i>et al.</i> (2013)	[12,13,22]
Riboswitch-regulated asRNA	Anti-PocR	Mellin <i>et al.</i> (2013)	[23]
<i>Trans</i>-encoded sRNAs			
	Rli31, Rli33-2, Rli50	Mraheil <i>et al.</i> (2011)	[10]
	Rli38	Toledo-Arana <i>et al.</i> (2009)	[12]
	Rli27	Quereda <i>et al.</i> (2014)	[34]
	LhrA	Christiansen <i>et al.</i> (2006)	[7]
CRISPR			
	rliB-CRISPR	Sesto <i>et al.</i> (2014)	[24]

Table 1. RNA-mediated regulatory mechanisms related to *Listeria* virulence

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