# Bacterial and cellular RNAs at work during Listeria infection.

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## Abstract/Summary

*Listeria monocytogenes*is 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. Theyregulate 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 invirulence and then the host miRNA response to*Listeria* infection.Finally, we discuss thepotential crosstalk between bacterial RNAs and host RNAregulatorymechanisms as new mechanisms of bacterial virulence.

## Keywords

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

## Introduction

The human pathogen *Listeriamonocytogenes* 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 feto-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 in to the mammalian cells by exploiting host cell receptorsand 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 impactingon the miRNA expression profiles of infected cells and tissues[1-4].

The mechanisms underlying mammalian and bacterial gene regulationsshare 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 andin controllingthe 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 RNAsfunction 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 apicture 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.

**The5'-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'-UTRshave evolved as efficient gene expression regulators that sense physicochemical signals (e.g. thermosensors and riboswitches), or can bind proteins and RNA regulatorsacting 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]. Agroup of 101 genes with an unusually long 5'-UTR (>100nt) includes10 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 familythat induces theexpression 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**,** whichadopts a stable stem-loop structure at a low temperature, therebyoccluding the SD sequence and preventing binding of the ribosome. When the temperature increases to37°C,the stem-loop melts into an alternative secondary structure, allowing the ribosome to access the SD sequence, leading to the translation of the*prf*A mRNA and to the subsequent induction of a number of virulence genes [20]. A second mechanism of *prf*A expression regulation involves a ***trans*-acting riboswitch-derived** element. Typically, riboswitches are 5'-UTR elements that, upon binding of aligand (tRNA, ions or metabolites),undergo conformational changes and affect the transcription or the translation of a nascent mRNA transcript.Riboswitch-regulated transcripts usually encode genesinvolved in the

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

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

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

13]. In other words, an excludon functions as a genomic togglewhere a single transcript governs the mutually exclusive expression of adjacent genes that generally have opposing functions. For example, an excludon regulatesthe 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 asRNAAspocR, which overlaps the gene encoding PocR, a transcription factor thatactivates 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 *poc*R, whereas in the absence of B12, AspocR is fully transcribed, thus negatively regulating PocR production. Interestingly, the negative regulation of *poc*Rexpression was observed whenAspocR was expressed *in trans,* indicating that it likely interferes with the transcription or translation initiation of *poc*R. 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

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

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

169 species *L. innocua*[13]. Comparative genomic studies of the two species have been
170 previously used to identify a number of *Listeria* virulence factors[1, 31], and it is thus
171 tempting to speculate that *L. monocytogenes*-specific sRNAs would play a role in
172 virulence. Indeed, nearly all sRNAs shown to have a role in virulence are absent from
173 thenon-pathogenic species. Among these, Rli33-2 and Rli50, when deleted, led to an
174 attenuated virulence phenotype in murine macrophage infection as well as in mouse and
175 butterfly larvae infection models[10]; similarly, a deletion mutant of Rli38 resulted in an
176 attenuated virulence phenotype in orally inoculated mice [12]. Another sRNA absent
177 from *L. innocua*Rli27has been recently shown to positively regulate the expression of
178 *lmo0514,* encoding an LPXTG surface protein enriched in the cell wall of intracellular
179 bacteria[32]. This regulation occurs by mechanism involving pairing of Rli27 with the
180 5'UTR of the *lmo0514* mRNA. Remarkably, *lmo0514* transcript is detected in two
181 variants, differing in length and in relative amount in extra- and intracellular bacteria.
182 Only the long version, more abundant in intracellular bacteria, contains the 5'-UTR
183 recognized by the Rli27,rendering this regulation possible only inside the host cell
184 (Quereda, et al.PLoS Genetics, *in revision*). Some sRNAs might have multiple target
185 genes, as shown in the case of LhrA which affects expression of nearly 300 genes and
186 directly regulates expression of *lmo0850, lmo0302* encoding proteins with an unknown
187 function and *chiA*encoding a chitinase[33, 34]. ChiA contributes to *Listeria* pathogenesis
188 [35].

189 It is worth mentioning that some *Listeria* sRNAs annotated as non-coding
190 transcripts encode putative open reading frames (ORFs) for small, often very basic
191 polypeptides, whose function is unknown. As reported for other species, these peptides
192 could act as signaling molecules involved in bacterial communication or might play a
193 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* geneselsewhere 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-encodedbi-functional enzyme harboring both3’ to 5’ exoribonuclease and 3’ polymerase activities[43]. The identification of RliB-CRISPR processing by PNPaserevealed 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 beimportant 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 whichpromotes 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-CRISPRreveals 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 inmultiple growth conditions relevant for the infectious processis 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 betodecipher the biological function of the many annotated, but so far unexploredncRNAs in *Listeria.* Altogether, recent research on *Listeria* RNA-mediated regulations, as well as theimpressive number of studies in other

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

245 **II. The mammalian miRNA response to *Listeria* infection**

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

259 As previously mentioned, miRNAsare involved in various physiological and
260 pathological processes. Their role during bacterial infections of animals has only
261 recently started to be investigated with several pioneering studies, e.g. in *Helicobacter*
262 *pylori, Salmonella enterica*and *Mycobacterium avium*[51-54]. The role of miRNAs during
263 *L. monocytogenes*infection has been addressed both in cultured cells[55, 56] as well as *in*
264 *vivo*in mice models[30, 54, 57]. Here, those studies will be presented in an order, which
265 may look awkward butfollows 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-200cwas affected by the presence of lactobacilli or the host microbiota, demonstrating the important role of intestinal bacteriain the modulation of the host miRNA response to infection [30, 57]. A singlemiRNA family wascommon 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]. ThemiRNA 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 asgenes 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 werepredicted 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 onthe production of interferon-γ (IFN-γ) by **natural killer (NK) cells**, which promotesthe 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-κB, 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 analysissuggested that all 5 miRNAs could potentially interact with

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

319 Thewholeinfectious process relies on the *Listeria* capacity to enter non-
320 phagocytic cells. During infection of **epithelial** cells, *Listeria* induces expression of miR-
321 155, miR-146b and miR-16 and decreases expression of let-7a1 and miR-145, all of
322 which are also implicated in the regulation of immune-related genes. Interestingly,
323 several major *Listeria* virulence determinants, the surface internalinsInlA and InlB as
324 well as the secreted toxin listeriolysin O (LLO), are implicated in the regulation of the
325 above-mentioned miRNAs [55]. Purified LLO could fully reproduce the *Listeria*-induced
326 miRNA expression profile whereas a*Listeria* deletion mutant for *inl*A and *inl*Bled to
327 decreased expression of miR-155, suggesting a putative role for internalins or *Listeria*
328 entry in miRNA regulation [55].

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

336 A significant effort has been made to identify numerous mammalian miRNAs,
337 both *in vivo* and in different cellular models, whose expression is regulated during
338 *Listeria* infection. Not surprisingly, the miRNA profile induced in the intestinal tissue is
339 different from thatinduced by a*Listeria* infection in different cell lines. Nevertheless, the
340 regulated miRNAssharesimilar 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 tothe intestinal miRNA response, miR-155 and miR-146 appear to beinduced 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-κB pathway,which regulates a number of genes critical to innate and adaptive immunity, cell proliferation, inflammation, and tumor development[64].

Althoughidentification of the miRNA profile during *Listeria* infection is clearly underway, a future challenge will be to decipher the molecular mechanism underlying themiRNA expression changesupon 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 anefficient infection and promote its survival in the host. The*Listeria*effectors known to be involved in these complex rolesincludeLLO, which forms pores, promotes escape from the vacuole, triggers histone modifications,other post-translational modifications and mitochondrial fragmentation, ActA which allows

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

371 While such RNA effectors have never been described in bacterial pathogens, and
372 while it was neverformally demonstrated that aspecific bacterial RNA is actively
373 delivered to the host cell, there is a strong logic supporting the existence of bacteria-host
374 RNA-mediated communication.First, many pathogenic bacteria as exemplified by
375 *Listeria,* enter the host cell and therefore haveaccess to different cellular compartments.
376 Second, they possessvarious systems of export/secretion that can secreteproteins, and
377 also nucleic acids.For instance, it has been shown that*Neisseria gonorrhoeae*can secrete
378 single stranded DNA by the type IV secretion system (T4SS) [68].In the case of *Listeria*, it
379 has been shown that it can secrete small nucleotides such as c-diAMP[69], and it was
380 recentlydemonstrated it can also release DNA and RNA during the infection of the host
381 cell [70]. Third, regulatory RNAs offer a general mechanism to interfere with
382 mammalian regulation.For example, a bacterial RNA could bind a host miRNA and
383 inhibit its function or it could mimic a mammalian miRNA, therebyovertaking the
384 miRNA-machinery andaffecting the expression ofthehost genes. As emphasized in the
385 preceding section, miRNAs are key components of the immune response [15, 64], which
386 makes them exceptional targets for such pathogen-induced manipulation.As shown
387 with*Listeria* and other pathogens, their expression is indeed significantly affected upon
388 infection. Alternatively, one should not exclude the possibility that RNA virulence

effectors mightaffect 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 (MCMV) 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, whichmimic host miRNA and bind to Argonaute 1 protein (AGO1), selectively silencinga 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* canreleasethe 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 RNAsare 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, whichinstead of a trimethylguanosine cap,consists of a triphosphate. RIG-I has been shown to recognizetriphosphorylated*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].Thesedata 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 andits potential benefit for the pathogen. A secreted RNAvirulence 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, whichhave 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 hostmiRNA 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 imaginethe exciting hypothesis that such secretedvirulence 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 bacteriumwhose virulence factors expression is controlled by RNA-mediated regulatory mechanisms.
- The expression of the main *Listeria*virulence regulator PrfA is regulated by an RNAthermosensor and a trans-acting SAM riboswitch.
- In *Listeria*, 13long asRNAs named excludon,whichregulate 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 theB12riboswitch-regulated asRNAAsPocR.
- There are more than 150 annotated sRNAs in *Listeria* with mostly unknown functions.Forsome of themit 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:**

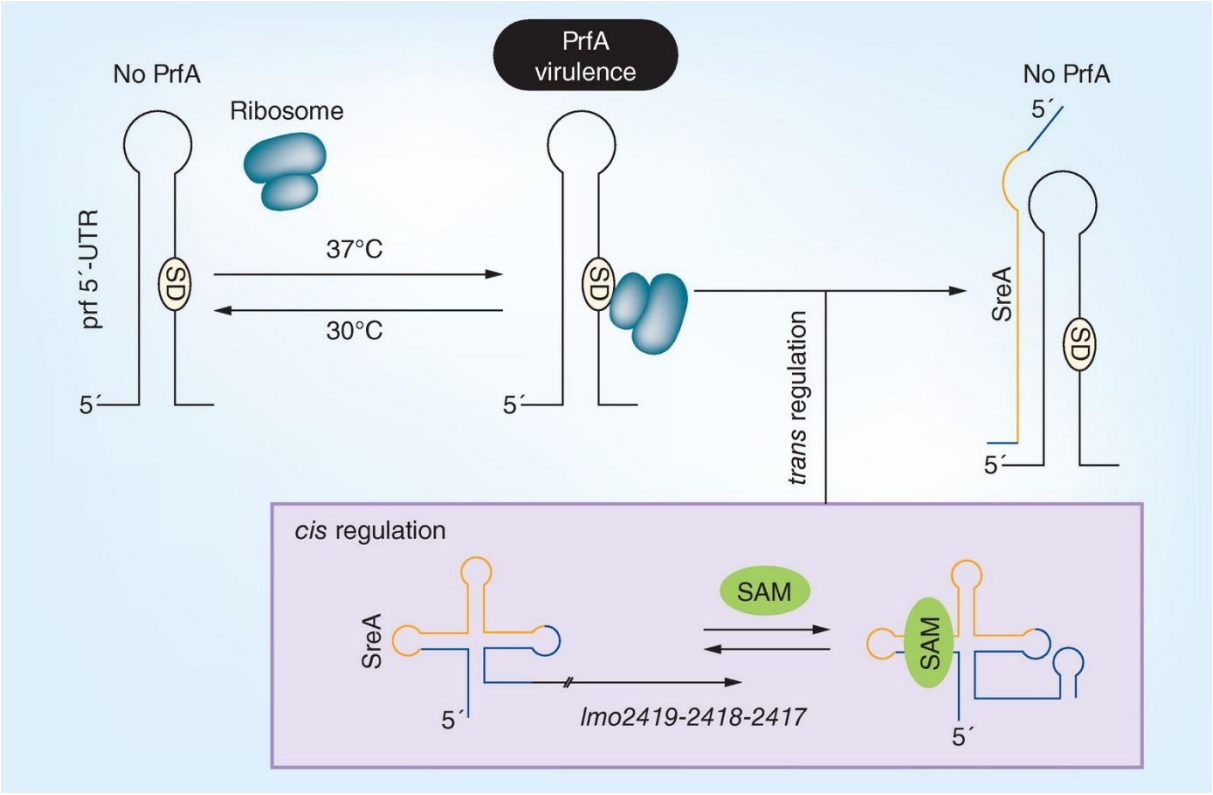
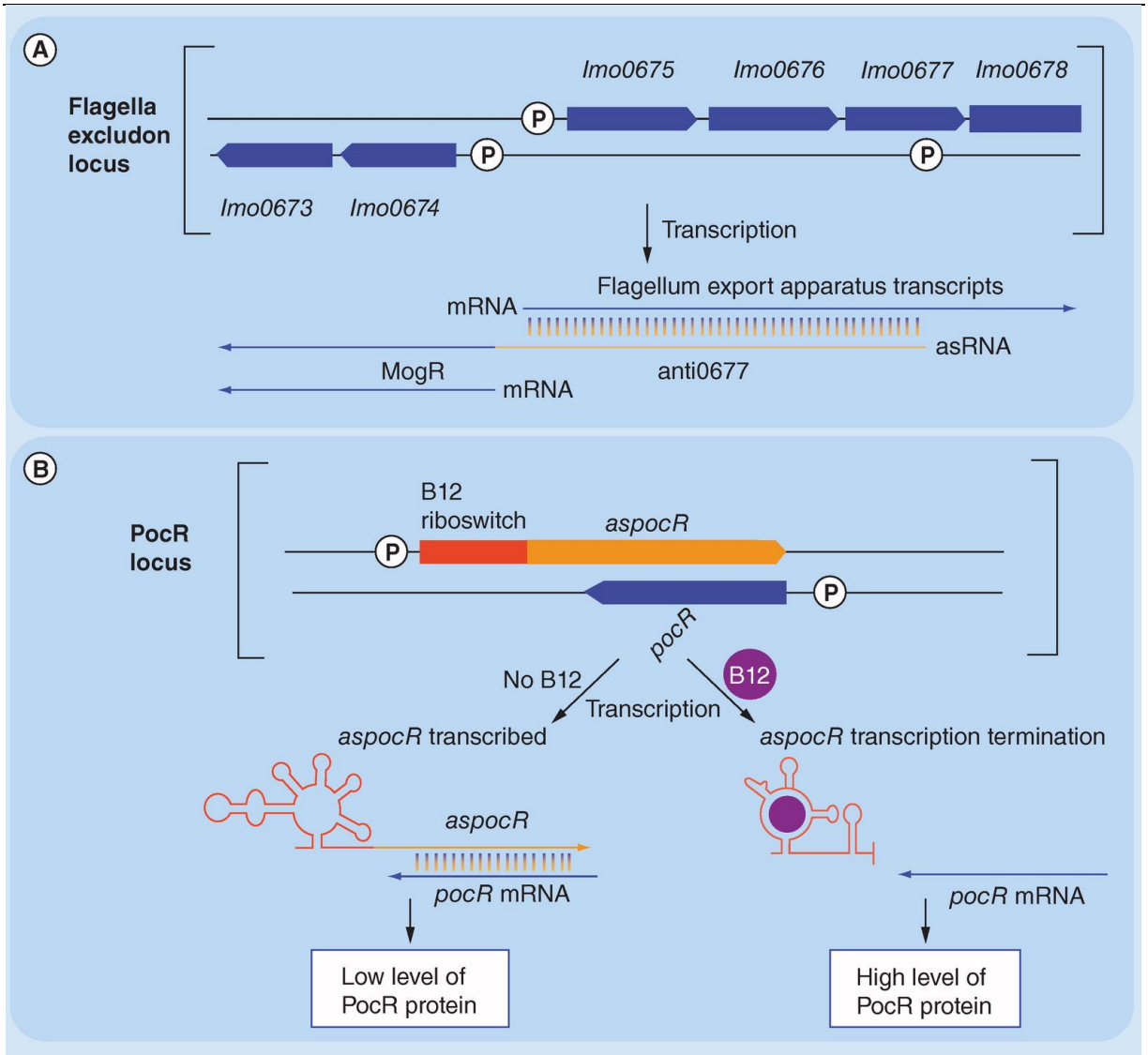



**Figure 1. 5'UTR-mediated regulation of PrfA expression.**

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 theS-adenosyl-methionine (SAM) riboswitch (SreA) interacts with the *prfA*5'-UTR and prevents the production of the PrfA protein.

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

718 **A)**Example of an excludon, where a long asRNA Anti0677 overlaps and serves as an
719 antisense regulator of *lmo0675*,*lmo0676* and *lmo0677* encoding FliN, FliP and FliQ,
720 respectively, which are components of the flagellum export apparatus, while
721 simultaneously encompassing the 5'-UTR and the mRNA of *lmo0674* encoding MogR,a
722 transcriptional repressor of the flagellum genes. The expression of Anti0677 is regulated
723 by sigmaB (σB, a stress-activated transcriptional regulator). Altogether, the excludon
724 ensures that by two mechanisms (inhibition mediated by the antisense component of
725 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 asRNAAspocR, 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.

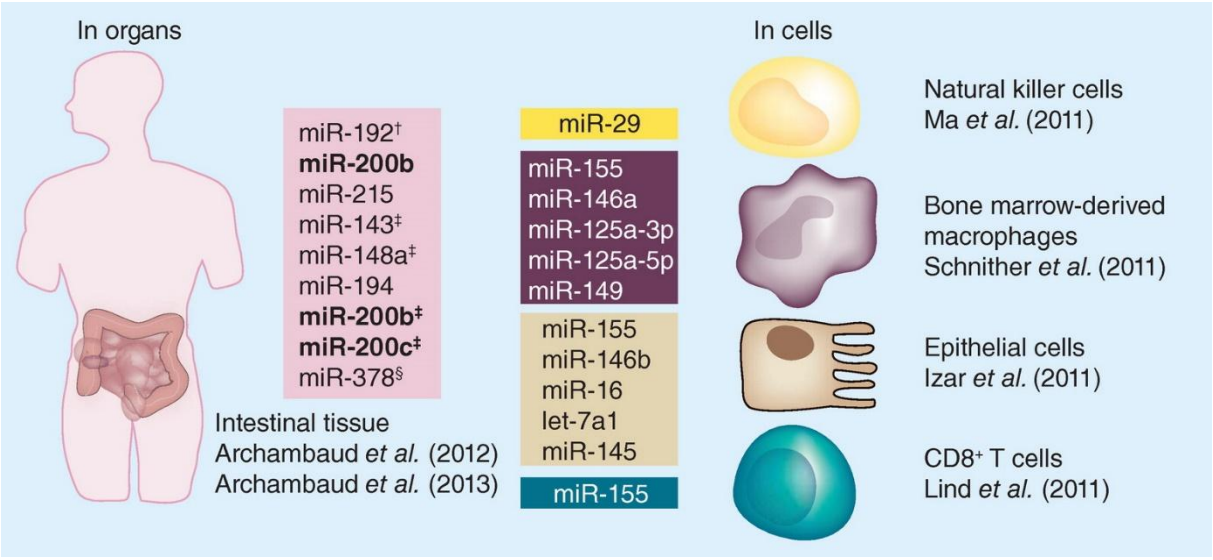


**Figure 3. Regulation of the host miRNA expression during *Listeria* infection.**

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.

744

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

745
746 **Table 1. RNA-mediated regulatory mechanisms related to *Listeria* virulence**
747