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Bacterial and cellular RNAs at work during Listeria infection.

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1 **Bacterial and cellular RNAs at work during *Listeria* infection**

2

3 **Abstract/Summary**

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

14

15 **Keywords**

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

18

19 **Introduction**

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

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

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

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

61

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

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

69 **The5'-untranslated region (5'UTR) of an mRNA**is located between the
70 transcriptional start site (TSS) and the translational initiation site. It harbours the Shine-
71 Dalgarno (SD) sequence to which the ribosome binds and initiates protein translation. In
72 prokaryotes, transcription and translation are coupled and therefore, many 5'-UTRshave
73 evolved as efficient gene expression regulators that sense physicochemical signals (e.g.
74 thermosensors and riboswitches), or can bind proteins and RNA regulatorsacting before
75 completion of the transcription/translation of the gene. The precise length of all 5'-UTRs
76 in the*Listeria* transcriptome has been recently determined by high resolution mapping
77 of the TSSs in a genome-wide manner [13]. Agroup of 101 genes with an unusually long
78 5'-UTR (>100nt) includes10 known *Listeria* virulence factors[13] among which some
79 have been extensively studied.

80 The main regulator that orchestrates the *Listeria* infectious process is PrfA
81 (Positive regulatory factor A), a transcription factor of the Crp/Fnr familythat induces
82 theexpression of major known virulence genes. Its expression is tightly regulated by two
83 RNA-mediated mechanisms operating at its 116 nucleotide long 5'-UTR (Figure 1). First,
84 the 5'-UTR of *prfA* mRNA is a **thermosensor**element**,** whichadopts a stable stem-loop
85 structure at a low temperature, therebyoccluding the SD sequence and preventing
86 binding of the ribosome. When the temperature increases to37°C,the stem-loop melts
87 into an alternative secondary structure, allowing the ribosome to access the SD
88 sequence, leading to the translation of the*prf*A mRNA and to the subsequent induction of
89 a number of virulence genes [20]. A second mechanism of *prf*A expression regulation
90 involves a ***trans*-acting riboswitch-derived** element. Typically, riboswitches are 5'-
91 UTR elements that, upon binding of aligand (tRNA, ions or metabolites),undergo
92 conformational changes and affect the transcription or the translation of a nascent
93 mRNA transcript.Riboswitch-regulated transcripts usually encode genesinvolved in the

94 biosynthesis of the molecule that regulates the riboswitch[21]. In the case of *Listeria,* the
95 short transcript of theSAM(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 *prf*A[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,

119 13]. In other words, an excludon functions as a genomic togglewhere a single transcript
120 governs the mutually exclusive expression of adjacent genes that generally have
121 opposing functions. For example, an excludon regulatesthe transcription of
122 flagellar/motility genes[12](Figure 2A). Flagella are important mediators of *Listeria*
123 pathogenicity[24]but at the same time, they are strong inducers of the host immune
124 response[25] and therefore, their tight regulation is crucial for *Listeria* survival during
125 infection.

126 The diversity of asRNA-mediated regulation is further illustrated by the
127 remarkable example of a **riboswitch-regulated asRNA** in *Listeria* [26](Figure 2B). A
128 vitamin B12-dependent riboswitch regulates the expression of the asRNAAspocR, which
129 overlaps the gene encoding PocR, a transcription factor thatactivates transcription of the
130 genes mediating propanediol catabolism (*pdu)* and vitamin B12 biosynthesis (*cob*).
131 Vitamin B12 is an important cofactor for the activity of diol-dehydratase, an enzyme
132 required for propanediol catabolism. In the presence of B12, the riboswitch terminates
133 prematurely AspocR transcription, allowing the subsequent expression of *poc*R, whereas
134 in the absence of B12, AspocR is fully transcribed, thus negatively regulating PocR
135 production. Interestingly, the negative regulation of *poc*Rexpression was observed
136 whenAspocR was expressed *in trans,* indicating that it likely interferes with the
137 transcription or translation initiation of *poc*R*.* Overall, this mechanism ensures that PocR
138 is produced uniquely when the B12 cofactor is available, allowing the subsequent
139 activation of the propanediol catabolism genes. Propanediol, together with the closely
140 related metabolite, ethanolamine,constitute important nutrient sources for bacterial
141 enteropathogens [27]. Recently, it was shown that during intestinal infection by
142 *Salmonella enterica*, use of ethanolamine as a carbon source enables the bacterium to
143 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].

194 **CRISPR/Cas systems (Clustered Regularly Interspaced Short Palindromic**
195 **Repeats)**provide bacteria and archaea with specific mechanisms of RNA-mediated
196 adaptive immunity against invading nucleic acids, i.e. viruses and conjugative plasmids.
197 Typically, CRISPR systems are composed of arrays of identical repeat sequences,
198 interspaced with non-repetitive variable spacers, coupled with clusters of CRISPR-
199 associated (*cas*) genes that are involved in all steps of CRISPR function.At the core of
200 CRISPR functionality are the spacers, short DNA segments originating from a foreign
201 DNA, which when transcribed provide a specific guide for CRISPR-mediated DNA/RNA
202 silencing of the corresponding invading virus or a plasmid[39]. *Listeria* species encode
203 three different CRISPR systems[40-42].CRISPR-I and/or CRISPR-II are present in
204 some*Listeria* strains and are always associated with*cas*genes. Their identified spacers
205 match uniquely*Listeria* bacteriophages.The third CRISPR,the RliB-CRISPR (previously
206 annotated as a sRNA named RliB)is present in all so far sequenced *Listeria* strains but is
207 never associated with a*cas*locus.However, both in the *cas*-less *Listeria* strains and in
208 those encoding a complete set of *cas* geneselsewhere in the genome(adjacent either to
209 CRISPR-I or CRISPR-II), the RliB-CRISPR is expressed and processed [42]. Surprisingly,
210 this processing is governed by the polynucleotide phosphorylase (PNPase), a genome-
211 encodedbi-functional enzyme harboring both3' to 5' exoribonuclease and 3' polymerase
212 activities[43]. The identification of RliB-CRISPR processing by PNPaserevealed a unique
213 role for this enzyme in bacterial "CRISPRology". Similarly to CRISPR-I and CRISPR-
214 II,RliB-CRISPR targets *Listeria* bacteriophages. Functional studies of RliB-CRISPR
215 showed it has a DNA-interference activity. Singularly, its activity requires that both
216 PNPase and the *cas*genes belonging to CRISPR-I are present in the genome. RliB-CRISPR
217 and CRISPR-I share a similar repeat sequence, suggesting they might share the same
218 enzymatic machinery required for their function[42]. Interestingly, RliB-CRISPR is

219 conserved in pathogenic *Listeria* species and its expression is significantly up-regulated
220 in bacteria isolated from the intestinal lumen of gnotobiotic mice and in bacteria grown
221 in the human blood. The*L. monocytogenes*EGD-emutant deleted for RliB-CRISPR
222 colonized liver of intravenously inoculated mice better than the wild type bacteria [12].
223 This phenotype was however opposite when mice were inoculated by the oral route
224 (our unpublished data), suggesting that RliB-CRISPR might beimportant during the
225 intestinal phase of the infection. Indeed, the human gut microbiome is rich in
226 bacteriophages and CRISPR systems are highly dynamic in such an environment [44,
227 45].Therefore, RliB-CRISPR contribution to *Listeria* virulence might be indirect by
228 impacting the bacterial survival challenged by bacteriophages. Additionally, during
229 *Listeria* intracellular infection, a temperate prophage is excised, reconstituting a
230 functional*comK* gene whichpromotes bacterial escape from the phagosome [46].
231 Whether RliB-CRISPR, CRISPR-I or CRISPR-II contribute to the control of the prophage
232 excision, remains to be examined. Altogether, RliB-CRISPRreveals the importance of the
233 interactions between bacteriophages and bacteria during saprophytic life and during
234 infection.

235 As a result of high throughput transcriptome studies a comprehensive overview
236 of the *Listeria* non-coding genome inmultiple growth conditions relevant for the
237 infectious processis publicly
238 available(http://www.weizmann.ac.il/molgen/Sorek/listeria_browser/). The functional
239 studies have revealed a broad diversity of regulatory mechanisms underlying the action
240 of individual RNAs. A future challenge will betodecipher the biological function of the
241 many annotated, but so far unexploredncRNAs in *Listeria.* Altogether, recent research on
242 *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.

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

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

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

306 In the following steps of the infection, *Listeria* is internalized by **macrophages**.
307 During the infection of bone marrow derived macrophages (BMDMs), *Listeria* induces
308 expression of 13 miRNAs among which miR-155, miR-146a, miR-125a-3p/5p and miR-
309 149 are the most significantly up-regulated [54]. This induction occurs already when
310 bacteria are in the phagosome and is mediated by MyD88, a universal adaptor protein
311 used by almost all Toll-like receptors (TLRs) to activate the transcription factor NF-κB, a
312 key regulator of the immune response to the infection. Indeed, miR-155 and miR-146
313 are known modulators of the immune response in macrophages [60, 61], whereas the
314 functions of miR-125a-3p, miR-125a-5p and miR-149 have not yet been described.
315 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),

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

354 Althoughidentification of the miRNA profile during *Listeria* infection is clearly
355 underway, a future challenge will be to decipher the molecular mechanism underlying
356 themiRNA expression changesupon infection as well as to identify their relevance for
357 the*Listeria*infectious process.

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

360 As emphasized in the introduction, *Listeria* has evolved a number of sophisticated
361 strategies to establish anefficient infection and promote its survival in the host.
362 The*Listeria*effectors known to be involved in these complex rolesincludeLLO, which
363 forms pores, promotes escape from the vacuole, triggers histone modifications,other
364 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

389 effectors mightaffect function of other host ncRNAs, such as lncRNAs,or could bind and
390 sequester host regulatory proteins as well as have other, yet unanticipated functions.
391 A direct evidence of such host-pathogen cross-kingdom RNA-mediated regulation
392 comes from some remarkable studies of viral and fungal pathogens. *Herpesvirus*
393 *saimiri*(HVS) and also cytomegalovirus (MCMV) express RNAs that interact and lead to
394 degradation of the host miR-27, consequently affecting the expression of miR-27 target
395 genes [71-73]. A fungal pathogen*Botrytis cinerea* expresses a set of sRNAs, whichmimic
396 host miRNA and bind to Argonaute 1 protein (AGO1), selectively silencinga subset of
397 host immunity genes [74].
398 It has been recently shown that during infection, viable *Listeria* can release
399 nucleic acids in the host cytoplasm [70]. As said above, this occurs also for other
400 pathogenic bacteria and is essential for generation of anti-microbial immunity[75].
401 Cytosolic *Listeria* canreleasethe second messenger c-di-AMP [69] as well as RNA/DNA
402 [70, 76]that are recognized by the sensors RIG-I, MDA-5 and STING, resulting in the
403 production of IFN. Bacterial RNAsare exceptionally good PAMPS (Pathogen Associated
404 Molecular Patterns – molecules associated with pathogens that are recognized by the
405 innate immunity) as they differ from the eukaryotic RNA by the nature of their 5’-end,
406 whichinstead of a trimethylguanosine cap,consists of a triphosphate. RIG-I has been
407 shown to recognizetriphosphorylated*Listeria* RNA [76]. More importantly, translocation
408 of RNA during the *Listeria* infection was visualized in the host cytosol using a sensitive
409 RNA fluorescence technique [76] and this translocation was shown to be dependent on
410 the activity of SecA-2 [70], an auxiliary protein secretion system that promotes secretion
411 of several virulence factors [77] as well as other genes whose expression is strongly
412 induced *in vivo* [78].Thesedata strongly indicate that the translocation of nucleic acids

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

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

420
421 **IV. Conclusions**
422
423 Decades of research led to the discovery of numerous *Listeria* molecular
424 strategies, whichhave been selected during the billions of years of pathogen-host
425 coevolution, to establish a successful infection. NcRNAs are versatile regulators
426 important for the *Listeria* virulence gene expression, metabolism regulation and the
427 interaction with the host.Similarly, eukaryotic miRNAs are potent regulators controlling
428 the expression of the human genome with an important accent on the immune response
429 regulation.This makes them a potent target for pathogen manipulation.Indeed, during
430 different phases of the *Listeria* infectious process,the hostmiRNA expression is
431 significantly altered. Similarity between prokaryotic and eukaryotic RNA-mediated
432 molecular mechanisms and the accessibility of the host RNA machinery to the
433 intracellular *Listeria* highlights a possibility of the interspecies RNA crosstalk between
434 the pathogen and the host.

435

436 **V. Future perspectives**

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

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

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

453

454

455 **Executive summary:**

456 *Listeria* regulatory RNA repertoire important for the virulence process

457 • *Listeria monocytogenes* is an invasive pathogenic bacteriumwhose virulence
458 factors expression is controlled by RNA-mediated regulatory mechanisms.

459 • The expression of the main *Listeria*virulence regulator PrfA is regulated by an
460 RNAthermosensor and a trans-acting SAM riboswitch.

461 • In *Listeria*, 13long asRNAs named excludon,whichregulate expression of genes
462 with opposite functions and act as fine-tuning regulatory switches, have been
463 identified.

464 • The *Listeria* vitamin B12 biosynthesis and propanediol catabolism, an important
465 nutrient during the intestinal phase of the infection, is controlled by the
466 transcription factor PocR. Expression of PocR is regulated by theB12riboswitch-
467 regulated asRNAAsPocR.

468 • There are more than 150 annotated sRNAs in *Listeria* with mostly unknown
469 functions.Forsome of themit has been shown a role in virulence.

470 • *Listeria* RliB-CRISPR system, which is processed with the help of chromosomally
471 encoded polynucleotide phosphorylase (PNPase), has a role in the virulence
472 process.

473 The mammalian miRNA response to *Listeria* infection

474 • During the infection, *Listeria* induces expression changes of the host miRNAs,
475 mainly regulating immune genes.

476 • The miR-200 family is specific to the intestinal miRNA response after orally
477 induced listeriosis and miR-155 and miR-146 appear to be induced by *Listeria* in
478 different cellular contexts.

479

480
481 A potential crosstalk of bacterial and mammalian regulatory RNAs during *Listeria*
482 infection

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

485

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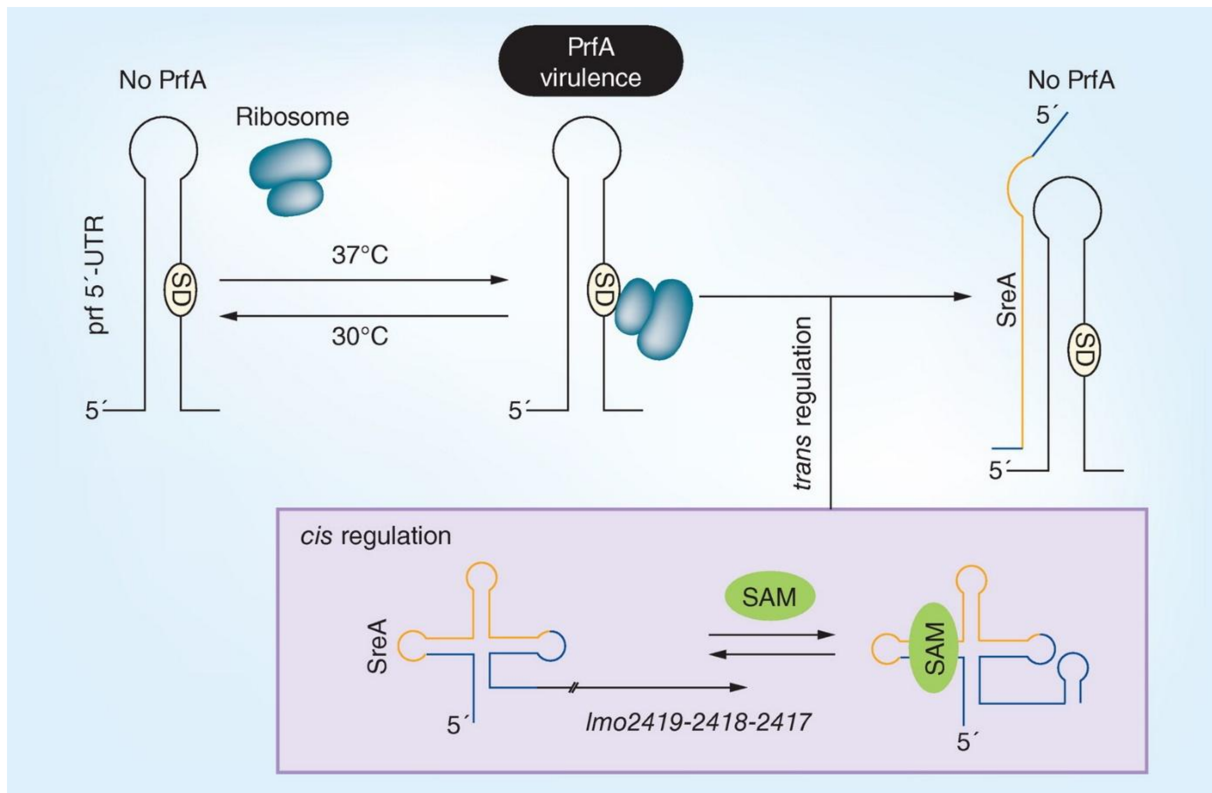
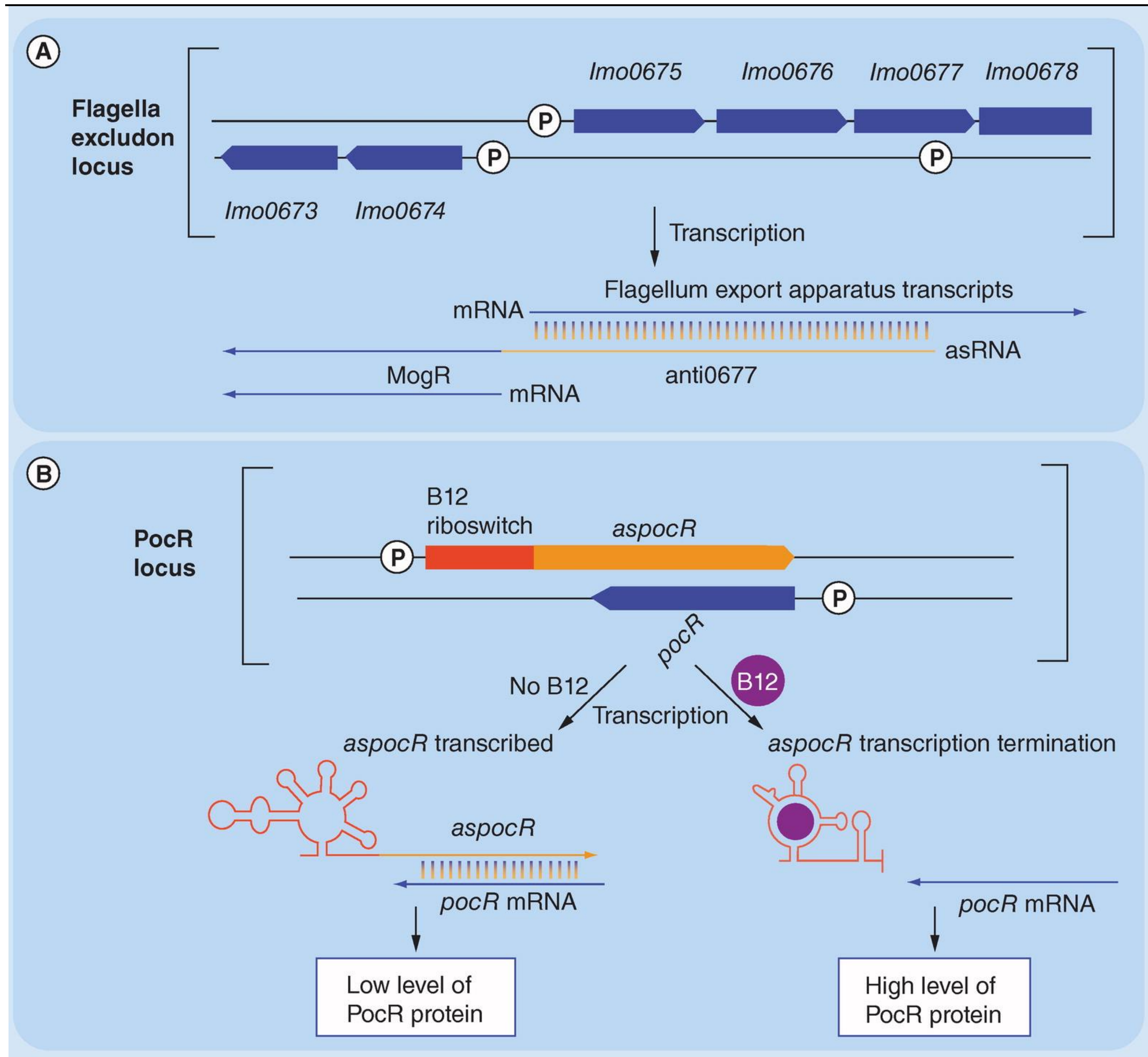
704 **Shows *Listeria* nucleic acids are released during the host cell infection *via* SecA2**
705 **dependent pathway.**
706
707 **Figures:**



708

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

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



716

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

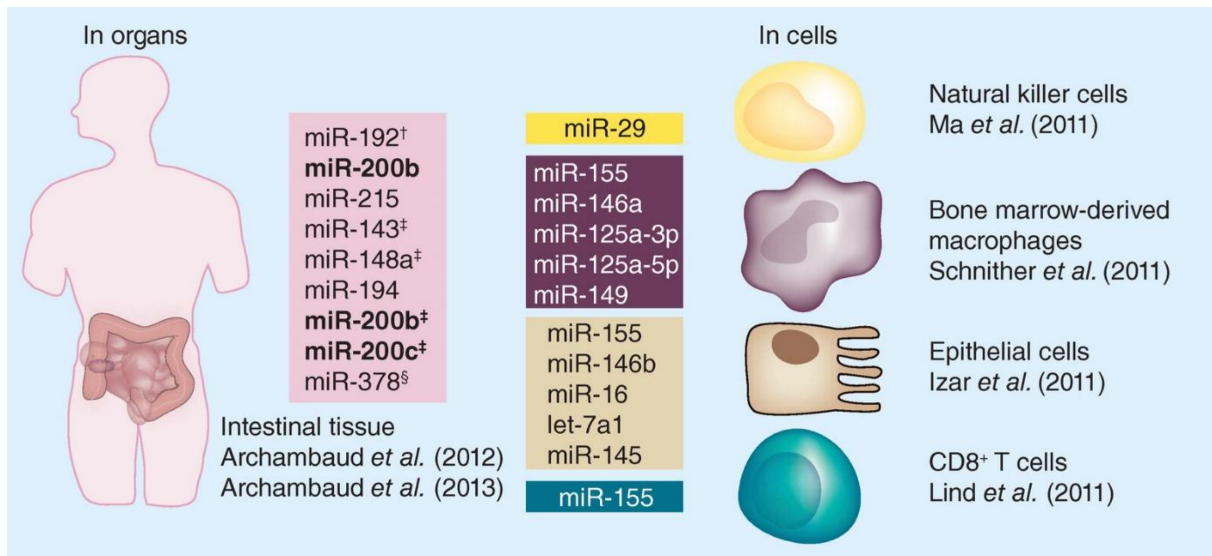
722 transcriptional repressor of the flagellum genes. The expression of Anti0677 is regulated

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)

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



732

733 **Figure 3. Regulation of the host miRNA expression during *Listeria* infection.**
734 Schematic representation of the significantly regulated miRNAs in the intestinal tissue
735 during orally acquired listeriosis (grey) and in infected cell lines (blue, purple, orange
736 and green). Highlighted are the miRNAs whose expression is modulated by the presence
737 of the host microbiota or lactobacilli: expression decrease upon *L. monocytogenes*
738 infection and expression increase upon treatment with *Lactobacillus* (*), expression
739 decrease only in the presence of microbiota upon *L. monocytogenes* infection (**),
740 expression decrease in the presence of microbiota and expression increase in the
741 absence of microbiota mice upon *L. monocytogenes* infection (***).In bold are miRNAs
742 detected to vary during different infections.

743

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