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RNA- and protein-mediated control of *Listeria monocytogenes* virulence gene expression

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Abstract

The model opportunistic pathogen *Listeria monocytogenes* has been the object of extensive research, aiming at understanding its ability to colonize diverse environmental niches and animal hosts. Bacterial transcriptomes in various conditions reflect this efficient adaptability. We review here our current knowledge of the mechanisms allowing *L. monocytogenes* to respond to environmental changes and trigger pathogenicity, with a special focus on RNA-mediated control of gene expression. We highlight how these studies have brought novel concepts in prokaryotic gene regulation, such as the 'excludon' where the 5'-UTR of a messenger also acts as an antisense regulator of an operon transcribed in opposite orientation, or the notion that riboswitches can regulate non-coding RNAs to integrate complex metabolic stimuli into regulatory networks. Overall, the *Listeria* model exemplifies that fine RNA tuners act together with master regulatory proteins to orchestrate appropriate transcriptional programmes.

Abbreviations and acronyms

ANTAR, *amiR* and *nasR* transcriptional antiterminator regulator; asRNA, antisense RNA; BCAA, branched-chain amino-acid; CAMP, cationic antimicrobial peptide; CRISPR, clustered regularly interspersed palindromic repeats; Crp, cyclic adenosine monophosphate receptor protein family; Csp, cold-shock domain- family protein; DNA, desoxyribonucleic acid; GSH, glutathione; lasRNA, long antisense RNA; LLO, listeriolysin O; mRNA, messenger RNA; ncRNA, non-coding RNA; ORF, open reading frame; PTS, phosphoenolpyruvate phosphotransferase system; RNA, ribonucleic acid; rRNA, ribosomal RNA; SAM, S-adenosylmethionine; SD, Shine-Dalgarno; sRNA, small RNA; TIS, translation initiation site; TSS, transcription start site; UTR, untranslated region.

Introduction

The foodborne pathogen *L. monocytogenes* is well known for its adaptation to a broad range of habitats, ranging from soil or wastewater to the mammalian intracellular space. This bacterial species has acquired the ability to survive and grow in a variety of temperature, pH or osmotic conditions, which allows it to colonize different niches. For instance, replication has been observed at temperatures close to zero, or up to 45°C.¹ *L. monocytogenes* can also use distinct types of motility; whereas flagella drive swimming motility in liquid media or swarming motility on semi-solid surfaces,² intracellular bacteria exploit actin polymerisation mechanisms to be propelled in the cytoplasm and from cell to cell.³ Upon a change in environmental conditions, bacteria can switch lifestyles through dramatic changes in their gene expression programmes, thus allowing saprophytic growth, stress response and resistance to extreme conditions, or triggering virulence properties.

Since the first completion of a *Listeria* genome,⁴ a number of transcriptomic studies have analyzed *Listeria* gene expression in a variety of conditions.⁵⁻⁸ In parallel, functional studies have revealed the role and impact of major transcriptional regulators coordinating the timing and extent of expression across gene networks in response to external cues.⁹ Among them, the transcription factor PrfA stands out as the master coordinator of a major virulence regulon.^{10,11} By improving the depth and resolution of transcriptome analysis, tiling arrays and high-throughput sequencing have more recently highlighted a significant impact of non-coding RNA (ncRNA)-mediated gene regulation in shaping bacterial gene expression.^{12,13}

We review here how studies on transcriptional reprogramming upon *L. monocytogenes* switch from saprophytism to virulence have unveiled original concepts and mechanisms of gene expression control, some of which can be generalized to other bacterial species. We will not discuss extensively the housekeeping transcriptome common to other prokaryotes; we rather aim at underlining how specific regulations of *L. monocytogenes* non-coding transcripts and virulence genes are coordinated during infection and contribute to pathogenesis.

1. Protein-mediated control of virulence gene expression

PrfA, the master regulator of *Listeria* virulence genes

The first identified and major regulator of virulence genes in *L. monocytogenes* is PrfA.^{14,15} This protein belongs to the broad family of cyclic AMP receptor protein (Crp) transcriptional regulators that can bind DNA as dimers on specific sites in gene promoters and activate transcription.¹⁶ PrfA was initially identified for its ability to stimulate the expression of the *hly* gene,¹⁴ encoding the pore-forming toxin listeriolysin O (LLO),¹⁷ and later on found to regulate major genes supporting intracellular life.¹⁸ *hly* is co-expressed together with a PrfA-dependent virulence cluster encompassing *prfA* itself, as well as the genes encoding *Listeria* secreted phosphatidylinositol-

The production and activity of PrfA are critical for, but not exclusive to, the intracellular stages of infection. Like for other members of the Crp family, PrfA activity is regulated by allostery. For more than twenty years, the nature of the small-molecule activator, which could specifically activate PrfA in the host cell cytosol, has remained elusive. PrfA activity was early known to be inhibited by environmental cellobiose, via an unknown indirect mechanism;²⁶ whereas a proteinase K-sensitive component present in human cell extracts could stimulate PrfA activity.²⁷ An elegant genetic screen using a *Listeria* strain unable to replicate when PrfA is active has recently revealed that the long-sought activating ligand was glutathione (GSH).²⁸ Intra-bacterial GSH levels depend on the uptake of this tripeptide from the host cell, and also on the intracellular production by the bacterial glutathione synthetase GshF. Of note, the expression of *gshF* is induced in the spleen of infected mice;²⁹ its regulators are unknown, and might for instance respond to sensing of redox conditions by the bacteria in their host.

PrfA activity has also been linked to the nature of the sugar sources available to the bacteria.³⁰ In the environment, the uptake of carbohydrates requires the phosphoenolpyruvate phosphotransferase system (PTS) before glycolysis; in contrast, in the host cell cytoplasm, bacteria can readily import phosphorylated hexoses via UhpT,³¹ and use them in the pentose phosphate pathway. It has been postulated that PrfA would be sequestered in an inactive state when the permeases in the PTS system are phosphorylated.³² Clear mechanistic evidence of this model is still missing, as well as a possible link between sugar uptake and GSH metabolism.

prfA transcription is also regulated by RNA elements, as further described below.

σ^B , a stress-responsive activator of *Listeria* virulence genes in the intestine

The transcription of a large number of *L. monocytogenes* virulence genes is additionally controlled by the stress-responsive, alternative sigma factor SigmaB (σ^B).^{33,34} σ^B is essential for the activation of an array of virulence genes in the intestine.³⁵ This regulon encompasses factors required for survival in the intestinal environment, such as Bsh, or crossing the intestinal barrier, such as InlA. Activation of the σ^B regulon is not restricted to *L. monocytogenes* pathogenic phase; as for other bacilli, it is induced in stationary phase when bacteria are cultivated in broth.³⁶ Importantly, the PrfA and σ^B regulons are significantly overlapping. This is partly due to the fact that σ^B regulates PrfA expression.³⁷ In addition, a number of genes are targets of both regulators.³⁸

VirR, a coordinator of surface components modification and antimicrobial resistance

Apart from these two main players, other transcriptional regulators contribute to the fine-tuning of gene expression during infection. Even though our goal here is not to describe their regulons extensively, we will briefly discuss how some of them contribute to transcriptional reprogramming. Among those, the two-component system VirR/S controls the expression of 17 genes, including its own operon.³⁹ A *virR* deletion mutant is defective for virulence in mice and entry in cultured epithelial cells. The expression of VirR/S and its regulated genes is enhanced in the spleen of infected mice.²⁹ In addition, VirR/S participates to resistance to a variety of antimicrobials and food preservatives.⁴⁰ In line with this, most of the characterized VirR/S targets contribute to the modification of bacterial surface components and/or provide resistance to antimicrobial peptides.⁴⁰ The *dlt* operon encodes proteins involved in D-alanylation of lipoteichoic acids and has been shown to be required for virulence;⁴¹ *lmo1695* and *lmo1696* encode respectively MprF, which can lysinylate phospholipids and confer resistance to cationic antimicrobial peptides (CAMPs),⁴² and a putative glycopeptide antibiotic resistance protein of the VanZ family;⁴³ last, the ABC transporter AnrAB is involved in resistance to the bacteriocin nisin, and to several other antibiotics.⁴⁴ VirR/S thus appears as a coordinator of bacterial protection against membrane and envelope damage, and against antimicrobials produced by its competitors or its host, or used in food preservation.

MogR, the repressor of motility genes

L. monocytogenes switches off its flagella genes during murine infection, extracellular growth at 37°C or intracellular growth.⁴⁵ The bacterial flagella play a role in two forms of the bacterial life; they allow the swimming motility of pelagic bacteria in liquid environment; they are also required for the colonisation of surfaces and biofilm formation.^{46,47} Expression of flagella genes is controlled by a complex network of transcriptional regulators. Among them, the motility gene repressor MogR and the glycosyltransferase and motility anti-repressor GmaR antagonize to allow flagellin production at 30°C, and repress it at 37°C.^{48,49} MogR acts as a repressor on the transcription of both flagella genes and *gmaR*. At low temperature, the anti-repressor GmaR interacts with MogR and inhibits it, thereby positively regulating its own expression, and allowing the transcription of the motility regulon. At higher temperature, the thermosensitive interaction between MogR and GmaR is disrupted, GmaR is degraded, and MogR represses the regulon. There is also evidence that motility is under a negative control by PrfA, probably via an indirect regulation.^{50,51}

CodY, a regulator of *Listeria* metabolism in host cells

Intracellular growth conditions also require the adaptation of carbon uptake and nitrogen assimilation to available sources. The nutrient-responsive regulator CodY coordinates the *de novo* synthesis of branched-chain amino-acids (BCAA) and sugar catabolism with the onset of virulence programmes and regulation of motility.⁵²⁻⁵⁴ Importantly, CodY activates the transcription of *prfA* by direct binding in its coding sequence, 15 nucleotides downstream of the start codon, and thereby stimulates the expression of virulence genes in response to low availability of BCAA (Fig. 1B).⁵⁵

2. RNA-mediated control of virulence gene expression

In addition to the action of transcriptional regulators, in-depth studies of the bacterial gene expression switches triggered by environment changes have highlighted a large variety of RNA-mediated regulatory mechanisms of bacterial gene expression.

Role of 5'-UTRs in virulence gene expression

5'-untranslated regions (UTR) have been shown to control the expression of several *L. monocytogenes* virulence genes at the post-transcriptional level. For instance, the 5'-UTRs of the *actA*, *hly*, *inlA* and *iap* are required for optimal expression of these virulence genes by affecting their stability or translation.⁵⁶

Regulation of mRNA stability by 5'-UTRs

The integrity of the 5'-UTR region of *hly* influences the abundance of messenger RNA (mRNA) as well as protein production, haemolytic activity and virulence, suggesting that these sequences participate in the stabilisation of the transcript.⁵⁷ Based on these findings, the 5'-UTR of the *hly* gene has been successfully used to enhance expression of genes in *L. monocytogenes* strains for research.⁵⁸ However, the stabilisation mechanism, which could rely on the protection from nucleases via the formation of secondary structures or binding of protective proteins, is still unknown.

The 5'-UTR of *inlA* also controls the expression of the *inlAB* transcript.⁵⁹ This regulation relies on the presence of a motif similar to a Shine-Dalgarno (SD) sequence, located immediately downstream of the transcription start site (TSS) and far upstream of the TIS. This sequence is proposed to act as a SD mimic named stability-SD (STAB-SD), which would stabilize transcripts by sequestering ribosomes.^{56,60} STAB-SD motifs are also found upstream of the *inlC* and *uhpT*

transcripts;⁵⁶ however, their role on the expression of these two virulence genes has not been explored.

Regulation of translation initiation by 5'-UTRs

Expression of the *iap* gene, encoding the invasion-associated murein hydrolase p60, is regulated via a post-transcriptional mechanism.⁶¹ The 5'-UTR of *iap* is thought to form a secondary structure masking the SD sequence and/or translation initiation site (TIS) and controlling translational initiation, depending on the binding of a yet-unknown ligand.

actA expression is also regulated via its 5'-UTR.⁶² The tight post-transcriptional control of ActA production is crucial for the bacterial ability to form comet tails and spread from cell to cell, independently of PrfA-mediated transcriptional activation in the cytosol. Note that the genomic region located 5' of *actA* has been annotated as a small RNA (Rli74, Fig1);⁶³ however, it has also been proposed that Rli74 represents in fact the long regulatory 5'-UTR of *actA*.¹² The status of this Rli remains so far unclear.

Relatively long 5'-UTRs in other virulence genes suggest that more candidates could be the object of similar modes of regulation, which remain to be characterized.^{12,35,56} Last, regulatory regions affecting translation can extend within the coding sequence of virulence genes. This was shown for *prfA*, whose 20 first codons enhance ribosomal binding to the SD region and are thus required for optimal translation initiation.⁶⁴

The *prfA* RNA thermosensor

Soon after the characterization of PrfA as an activator of the expression of *hly*,¹⁴ the expression of PrfA-dependent virulence genes was found to be thermoregulated in *L. monocytogenes*.⁶⁵ Indeed at low temperatures, *prfA* is transcribed but very low amounts of PrfA proteins are produced.²⁶ The mechanism of this regulation relies on a thermosensor, consisting of a secondary structure in the 5'-UTR of *prfA* transcripts that controls translation depending on the temperature (Fig. 1C).⁶⁶ At environmental temperatures (30°C or below), the SD sequence of *prfA* is sequestered into a closed stem-loop structure, which blocks the access of the small ribosomal subunit and thereby inhibits translation initiation. During infection of warm-blooded organisms, the temperature rise to 37°C opens the stem-loop and unmask the TIS. PrfA is then produced, which results in the transcription of the PrfA regulon; by doing so, PrfA also stimulates its own transcription, by acting on the *plcA* promoter, thus generating a bicistronic *plcA-prfA* transcript (Fig. 1A).

The example of the PrfA thermosensor is a good illustration that RNA structures are highly sensitive to temperature, and that their folding or unfolding can affect their function. For organisms that can live in a wide range of temperatures like *Listeria*, the ability to resolve RNA structures is crucial to resist cold shock and allow cold growth; in line with this, mutants in helicase genes usually display cold-sensitive phenotypes.⁶⁷ Two families of RNA binding proteins participate in cold-stress adaptation in *Listeria*: the cold-shock domain family proteins (Csps) CspA, CspB and CspD, and the DExD-box, ATP-dependent RNA helicase family proteins CshA, Lmo1246, Lmo1450 and Lmo1722.^{68,69}

Expression of Csps and helicases is induced during cold stress at 3 or 4°C.^{68,70,71} In addition, deletion mutants of *cspA*, *cspD*, *cshA*, *lmo1450* or *lmo1722* are impaired in growth and motility at low temperature.^{68,69,71} Lmo1722, and to a lesser extent CshA and Lmo1450, are involved in the maturation of the large ribosomal subunit, processing of 23S ribosomal RNA (rRNA) and maintenance of active translation.^{69,72} The helicases are also required for PrfA-dependent expression of virulence genes *actA*, *hly*, *inlA* and *inlB*, and for haemolytic activity.^{69,73} However, the expression of PrfA itself is not affected in helicase mutants, suggesting that *Listeria* helicases promote PrfA activity on its targets.

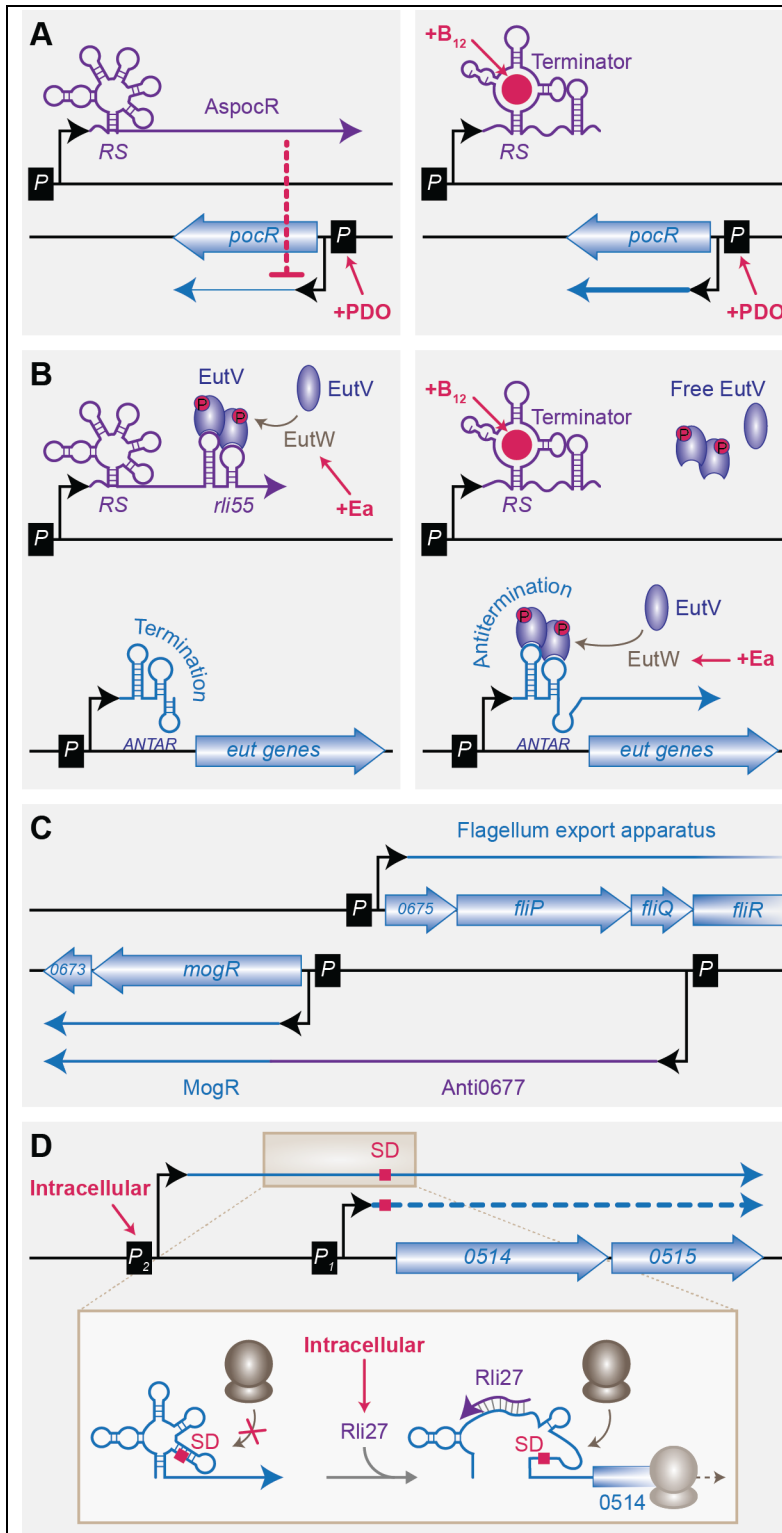


Fig. 2. Novel RNA-mediated regulations of *L. monocytogenes* gene expression.

A. Control of AspocR by a B₁₂ riboswitch, adapted from Cossart and Lebreton.²⁵ (Left) In absence of B₁₂, full-length AspocR is produced and inhibits *pocR* expression. (Right) In presence of B₁₂, the transcription of AspocR is terminated prematurely by the B₁₂ riboswitch (RS), and transcription of *pocR* is allowed, provided that propanediol (PDO) is also present in the medium. **B. Sequestration of an antiterminator by a B₁₂ riboswitch**, inspired from Mellin *et al.*⁷⁴ In presence of Ethanolamine (Ea), the antiterminator EutV is phosphorylated by EutW and activated. (Left) In absence of B₁₂, full-length Rli55 is produced and sequesters active EutV. The transcription of *eut* genes is prevented by early termination at the ANTAR elements. (Right) In presence of B₁₂, the transcription of Rli55 is terminated prematurely by the B₁₂ riboswitch. Free phosphorylated EutV dimers can then bind the ANTAR element and mediate antitermination, allowing the expression of *eut* genes.

C. The *mogR-fli* locus excludon, adapted from Cossart and Lebreton.²⁵ *mogR* is encompassed by two transcripts. In the longest one, the 5'-UTR of upstream of MogR coding sequence also acts as an antisense RNA, Anti0677, overlapping the *fli* operon. The two divergent transcriptional units encode proteins of opposite functions: MogR is a trans-

riptional repressor of flagellum genes, whereas Fli proteins participate in the flagellum export apparatus. **D. Rli27 allows the intracellular-specific translation of an alternative transcript**, inspired from Quereda *et al.*⁷⁵ Extracellular bacteria express mainly *lmo0514-lmo0515* from the constitutive P₁ promoter. An alternative, long mRNA is produced in low amounts from P₂, but ribosomes cannot access the Shine-Dalgarno sequence (SD). In cells, the P₂ promoter is activated, and Rli27 is expressed. Base-pairing of Rli27 with the 5'-UTR of the long transcript allows ribosome binding and translation to proceed.

Riboswitches

Riboswitches consist in short RNA sequences that change conformation upon binding of a dedicated ligand, and adjust the expression of certain operons according to the availability of specific metabolites. Typical riboswitches are parts of the 5'-UTRs of genes, where they can for instance act as transcription terminators or anti-terminators, or control the access of the small ribosomal subunit to the SD sequence.⁷⁶ The structure of these motifs is highly conserved across species, allowing their rather accurate prediction throughout genomes and their annotation in databases like Rfam (<http://rfam.xfam.org>).⁸¹

The genome of *L. monocytogenes* encompasses at least 55 validated or candidate riboswitches, responding to 13 distinct ligands.¹² The identification of the 'on' and 'off' states of 40 of these riboswitches by transcriptome analysis using tiling arrays has pinpointed that functional riboswitches can also be located in 3'-UTRs. For instance, a lysine riboswitch (LysRS), which controls the transcription of the downstream gene *lmo0798* encoding a lysine transporter, also acts as a transcription terminator for the upstream gene *lmo0799*.³⁵ Likewise, several other riboswitches from different families were proposed to act as transcription terminators.

A riboswitch-regulated antisense RNA

A vitamin B₁₂-dependent riboswitch, positioned downstream of the *lmo1149* gene, is in a convergent orientation with the gene *lmo1150* encoding a transcriptional regulator, PocR (Fig. 2A). Contrary to the initial assumption that this riboswitch was a transcriptional terminator of *lmo1149*, the riboswitch controls the transcription of an antisense RNA to *pocR* (AspocR), and thereby positively regulates PocR expression when vitamin B₁₂ is available.⁷⁷

The transcriptional factor PocR is required for the expression, in presence of propanediol, of two operons: *pdu*, which governs propanediol utilisation, and *cob*, which is involved in vitamin B₁₂ biosynthesis. The two functions are intimately linked, due to the need of B₁₂ as a cofactor for propanediol catabolism. The B₁₂-dependent antisense-mediated regulation of *pocR* ensures that *pdu* genes are expressed only when the substrate and the cofactor are available together. When propanediol is present but in absence of B₁₂, PocR expression is partially repressed by AspocR. These low levels of PocR are however sufficient to stimulate the expression of *cob* genes, allowing the synthesis of the cofactor while *pdu* genes are repressed.

Sequestration of an antiterminator by a riboswitch-regulated ncRNA

The investigation of the role of other B₁₂-regulated ncRNAs in *L. monocytogenes* has shed light on a new type of RNA-mediated regulation in the ethanolamine utilisation operon (*eut*).⁷⁴ This operon is activated in the presence of ethanolamine via a two-component system, EutVW, where EutW is a sensor kinase, and EutV acts as an antiterminator. In presence of ethanolamine, EutW activates EutV. This is however not sufficient to upregulate the operon, which requires ethanolamine and vitamin B₁₂ to be transcribed. This can occur due to the presence, upstream of the operon, of a B₁₂-riboswitch controlling the expression of a small RNA (sRNA), Rli55 (Fig. 2B).

In the absence of B₁₂, full length Rli55 is transcribed; it contains an EutV binding site, consisting of a structural motif similar to ANTAR (*amiR* and *nasR* transcriptional antiterminator regulator) elements. Rli55-ANTAR sequesters EutV and thereby prevents the transcription of *eut* genes. In the presence of B₁₂, the riboswitch terminates the transcription of Rli55 upstream of the ANTAR element. EutV is no longer titrated and can bind ANTAR elements in the transcripts of its target genes, thus allowing their antitermination. As for propanediol catabolism, enzymes of the ethanolamine utilisation pathway use vitamin B₁₂ as a cofactor; here again, the dual regulation of the operon ensures the optimal timing of gene expression with regards to the availability in metabolites. A similar mechanism has been identified in *Enterococcus faecalis*, where the small RNA EutX

sequesters EutV in absence of B12, whereas it is terminated by a B12 riboswitch when the concentration of this vitamin increases.⁷⁸

Interestingly, the three operons *pdu*, *cob* and *eut* are significantly up-regulated in the intestinal lumen of mice during orally-acquired listeriosis.⁷ This induction implies that the gut environment provides *L. monocytogenes* with the metabolites required for these pathways. Several other enteropathogens, such as *Salmonella enterica*, *Enterococcus faecalis* and *Clostridium perfringens* harbour similar operons, whereas these functions are deficient in most bacterial species from the microbiota;⁷⁹ this has been proposed to confer a metabolic advantage to pathogenic species in competition with the gut flora. In agreement with this hypothesis, competition experiments between lactobacilli and *L. monocytogenes* during intestinal infection led to an even stronger induction of the three operons,⁷ suggesting that *Listeria* can adjust its carbon metabolism in presence of commensal competitors towards unshared carbon sources.

Regulation of antibiotic resistance by conditional transcription termination

Our current repertoire of known bacterial riboswitches and other conditional, premature transcription terminators (attenuators, antiterminators) is far from being exhaustive. A novel, unbiased approach was recently developed for *ab initio* discovery of regulated, premature termination events, by systematic sequencing of RNA 3'-ends (term-seq).⁸⁰ In addition to validating known or predicted conditional terminators in *Bacillus subtilis*, *L. monocytogenes* and *E. faecalis*, this work has revealed premature termination events that can be alleviated by antibiotics. In *L. monocytogenes*, termination of Rli53 and Rli59, in the 5'-UTR of *lmo0919* and *lmo1652* respectively, is hampered by sub-lethal doses of translation-inhibiting antibiotics. Rli53 behaves as a terminator/anti-terminator structure, specifically responding to lincomycin. In presence of the antibiotic, Rli53 switches to an open state and allows transcriptional read-through into the downstream ORF. The latter encodes an ABC transporter providing resistance to lincomycin.

By applying term-seq to oral commensals submitted to short antibiotic treatment, similar patterns of regulation of antibiotic-resistance genes in gram-positive members of the oral microbiota could be identified, illustrating that conditional transcription termination represents a general feature in the set-up of antibiotic resistance. The use of this pipeline should allow the identification of unknown regulators responding to diverse ligands in the coming years, and provide a more accurate overview of the variety of RNA-mediated mechanisms used by bacteria to adapt to their environment. This expanding toolkit could also be used in the design of specific biosensors in diagnostic or industry.⁸¹

Cis-regulation by antisense transcription

Cis-antisense RNAs in *Listeria monocytogenes*

Pioneering transcriptomic analysis based on the use of macroarrays with a limited number of oligonucleotide probes per transcripts allowed the differential analysis of *L. monocytogenes* gene expression, depending on growth conditions, strains or mutations in regulators.⁸² The increase in resolution provided first by tiling arrays, soon followed by high-throughput sequencing of full-length mRNAs or 5'-ends, has allowed the precise mapping of TSSs and transcript lengths, and the identification of novel transcriptional units.^{35,36,83}

The resulting thorough characterisation of *Listeria* transcripts have revealed an unexpected wealth of ncRNAs, a number of which actively participate in co- or post-transcriptional control of gene expression. Among them, around a hundred sequences are transcribed in opposite orientation to another transcript.¹³ These *cis*-encoded antisense RNAs (asRNA) are potential negative regulators of gene expression on the opposite strand. We will not detail here the various possible mechanisms of antisense-RNA mediated repression, which have been extensively covered by

others,⁸⁴ and include transcriptional interference, transcriptional attenuation, alteration of transcript stability, and translational inhibition. *L. monocytogenes* asRNAs vary in length from 30 to several thousands of nucleotides. They can be antisense to known open reading frames (ORF), or occur due to the overlap of 3'-UTRs of convergent genes, or 5'-UTRs of divergent genes.

mRNAs with a dual function: the excludon concept

An original class of divergent transcriptional units was shown to rule the expression of operons encoding opposite functions, and consequently named “excludon”.⁸³ Typically, an excludon corresponds to a situation where the long 5'-UTR of one transcript overlaps with the coding sequence of the upstream unit, which is transcribed in the other direction (Fig. 2C). The 5'-UTR thereby acts as a long antisense RNA (lasRNA) and inhibits the expression of the divergent operon, while its own downstream sequence can be translated. This situation where a transcript can exert a dual function, as a messenger RNA and a non-coding antisense RNA, has first been described at the *mogR* locus.³⁵ As indicated above, MogR is a negative regulator of flagella synthesis genes, which are induced in the environment and repressed during infection. Among these, the *fli* operon, encoding proteins required for the synthesis of the flagellum export apparatus, is transcribed opposite of the *mogR* operon. The latter can be transcribed from two distinct promoters: a constitutive promoter is located just upstream of *mogR* coding sequence; an additional σ^B -dependent promoter is located within the coding sequence of *fliQ*, and thus gives rise to a long 5'-UTR antisense to *fliQ* and *fliP*. Abrogation of the long *mogR* transcript, by either mutation in the σ^B -dependent promoter or deletion of *sigB*, enhances the expression of *fliP*.

High resolution transcriptomics has extended the concept to 13 other pairs of divergent operons throughout the genomes of *L. monocytogenes*, involved in a variety of functions such as permeases versus efflux pumps, utilisation of distinct carbon sources, and nucleic acids metabolism.^{8,85} Similar divergent operons, where the 5'UTR of one gene extends into the coding sequence of the upstream gene, and thus possibly forming excludons, were identified in other members of the *Listeria* genera, as well as in more distant bacterial species.^{85,86}

Trans-acting functions of small RNAs

The *Listeria* genome encodes many ncRNAs that share a partial complementarity with targets located elsewhere in the genome, and can regulate them in *trans*. More than 150 putative *trans*-acting sRNAs, named Rli, have been annotated to date.^{13,87} The expression of several of them is enhanced or repressed in a mouse model of listeriosis or intracellularly; in addition, deletion mutants of *rliB*, *rli31*, *rli33-1*, *rli38* and *rli50* impair virulence in this mouse model of listeriosis, in *Galleria mellonella*, or during growth in macrophages, suggesting a regulatory function in virulence.^{35,63} However, the functions of most *Listeria* sRNAs remain uncharacterized.

Hfq-dependent small RNAs control chitinase activity

In Gram-negative bacteria, most *trans*-acting sRNAs form a complex with Hfq, a RNA chaperone required for their function.⁸⁸ This does not seem to be the rule in Gram-positive bacteria, including in *L. monocytogenes*, where Hfq is dispensable for the stability of several sRNAs.⁸⁹ Nevertheless, a Δhfq mutant is affected in stress tolerance, and in virulence in mice.⁹⁰ Furthermore, three sRNAs could be co-immunoprecipitated with Hfq: The *Listeria* Hfq-binding RNAs LhrA (Rli17), B (Rli14) and C (Rli4).⁹¹ Among them, LhrA directly controls the expression of *lmo0302*, *lmo0850* and the chitinase gene *chiA*. Hfq is required for the base-pairing of LhrA with *lmo0850* mRNA, which induces the degradation of its target.⁹² LhrA and Hfq also interfere with the recruitment of the ribosomes to *lmo0302* and *chiA*, thus repressing their translation.⁹³ LhrA thereby acts as a repressor of chitinolytic activity, which contributes to *L. monocytogenes* pathogenicity in mice.⁹⁴

A small RNA antagonizes a global post-transcriptional regulator

The virulence-associated sRNA Rli31 was recently found to bind the 5'-UTR of *spoVG* mRNA, as well as SpoVG itself, a RNA-binding protein and a global post-transcriptional regulator of motility, carbohydrate metabolism and virulence genes.⁹⁵ Deletions of *rli31* and *spoVG* have opposite phenotypes for lysozyme resistance and virulence in a mouse listeriosis model and are mutual suppressors, suggesting that their functions are antagonistic. The molecular mechanisms underlying these opposite effects will require further investigation.

A small RNA stimulates the translation of an alternative transcript

Another sRNA, Rli27, can base-pair within the 5'-UTR region of *lmo0514*, encoding a bacterial cell-wall protein (Fig. 2D).⁷⁵ *lmo0514* can be encoded as two transcripts differing in their 5'-UTR; the shortest one is constitutive, whereas the longest one as well as Rli27 are induced in intracellular bacteria. Binding of Rli27 to the long 5'-UTR unmasks the SD sequence and promotes translation. The two levels of regulation, transcriptional via the two distinct promoters, and translational via Rli27, act in synergy to sharply enhance the production of Lmo0514 protein when bacteria reach the host cytosol.

Short transcripts generated by a riboswitch can act in *trans*

In addition to the *cis*-regulating activity of riboswitches on transcriptional regulation, they can give rise to small RNAs with a *trans*-acting function. This was shown for two S-adenosylmethionine (SAM) riboswitches, SreA and SreB.⁹⁶ SreA controls the expression of genes encoding an ABC transporter (*lmo2419* to *lmo2417*) during the switch from exponential (high intracellular concentration of SAM) to stationary phase (low SAM concentration). SAM binding to the RNA aptamer causes early transcription termination after 229 nucleotides. This short SreA riboswitch transcript can base-pair, in *trans*, with the 5'-UTR region of *prfA* mRNA, mask the SD sequence and impede translation initiation (Fig. 1C). Conversely, PrfA is a transcriptional activator of SreA. The short riboswitch-containing RNA is thus part of a negative feedback loop on the expression of the main virulence gene regulator.

Similarly, a second short transcript released by a SAM riboswitch, SreB, can bind *prfA* mRNA and prevent its translation. Altogether, these early-terminated transcripts add an additional layer of regulation to the subtle network restricting *prfA* expression in the host.

PNPase binds and processes an atypical Rli/CRISPR

CRISPRs represent a widespread class of genetic loci in bacteria, which can provide acquired immunity against bacteriophages and conjugative plasmids.⁹⁷ They consist of a series of repeats and spacers of fixed size acquired from an invading DNA. These loci can be transcribed, processed by cleavage within the repeats, and loaded into an effector protein complex. Base-pairing of a specific CRISPR spacer RNA sequence to a cognate nucleic acid drives the endonucleolytic activities of the effector complex to eliminate invading agents.

Listeria RliB is an atypical member of the CRISPR family, conserved at the same genomic locus in all analysed *L. monocytogenes* genomes and also in other *Listeria* species.⁹⁸ The deletion of *rliB* impairs virulence in mice.³⁵ The processing and activity of CRISPRs usually depend on *cas* (CRISPR-associated) genes, transcribed in the immediate vicinity of CRISPR loci. In contrast, no *cas* genes are found in the neighbourhood of RliB.⁹⁸ Biochemistry experiments have shown that RliB is stably bound by, and also a substrate of, *Listeria* polynucleotide phosphorylase (PNPase), which is likely responsible for its processing into a mature form. Last, in *L. monocytogenes* strains encoding a complete CRISPR-*cas* system elsewhere in the genome, RliB can hamper, albeit

modestly, the uptake of exogenous DNA corresponding to its spacer sequences, suggesting that Cas proteins from another locus can complement a defective one.

Conclusions

Upon infection, the timely set-up of virulence programmes by *Listeria monocytogenes* relies on a wide variety of control mechanisms acting at all steps of gene expression as listed above. Among the identified regulations, a striking observation is the extreme versatility of mechanisms controlling PrfA regulon. *prfA* transcription is activated by σ^B in response to stress, by CodY in response to BCAA depletion, and by PrfA itself in a positive feedback loop (Fig. 1). Its translation is controlled by temperature due to a thermosensor in its 5'-UTR region, and tuned by the trans-acting riboswitches SreA and SreB. PrfA activity is dependent on the binding of glutathione, the levels of which increase when bacteria reach the cytosol. We have also mentioned that PrfA target genes *actA*, *hly*, *inlA* and *inlB* are the object of specific post-transcriptional regulation. This apparent wealth of mechanisms might reflect the fact that the PrfA regulon has been more studied than others, due to its primary role in virulence. Nonetheless, it stresses the crucial importance of a fine-tuning of the expression of virulence genes, limiting their effects to the adequate time and place. In agreement with this, a mutant in *prfA* which leads to constitutive PrfA activity, enhanced invasion in cell cultures and hyper-virulence in mice (PrfA*), displays impaired growth in rich medium.⁹⁹ In addition, the cross-coordination of the PrfA regulon with other pathways attests of the ability of invasive bacteria to integrate multiple signals, in order to adapt their metabolism, growth and pathogenic potential to their new environment.

Further studies will be required to fully document the molecular basis of virulence gene expression regulation. For instance, the precise post-transcriptional mechanism of regulation by many 5'-UTRs is still unknown; most excludons and their role in adaptation to environmental conditions are uncharacterized; our understanding of the influence of *in vivo* cues and commensals on *Listeria* transcriptome remains fragmentary; research on the comparative role of transcriptional regulation in virulence in between *L. monocytogenes* strains is only in its infancy, and could provide a ground for pathogenic variability among closely-related isolates.¹⁰⁰

Beyond gene expression regulation, the denomination small RNA does not necessary mean non-coding RNA. Among the remaining sRNAs to be characterized in *Listeria*, 19 might prove to be coding for small peptides.¹² Last, an emerging theme in the field focuses on the ability of bacterial mRNAs and ncRNAs to be modified, like what was long known for rRNAs and transfer RNAs (tRNAs).¹⁰¹ Base edition offers a potent lever for flexibility in transcript functions, the impact of which on bacterial physiology and virulence is likely underestimated, and will probably be better assessed in the coming years.

We have discussed here the role of transcription reprogramming and non-coding RNA in *Listeria monocytogenes* virulence. An intriguing hypothesis is whether RNA molecules could also play a role as virulence effectors. In theory, this is not excluded, since short RNA fragments can be secreted by *Listeria* into the cytoplasm of infected macrophages or epithelial cells, depending on the SecA2 secretion system.^{102,103} However, so far only a role as activators of the innate immune RNA sensor RIG-I was identified for these RNAs. An active function of these secreted nucleic acids is still to be found.

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