Regulating Bacterial Virulence with RNA

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Shortened running title: regulating Bacterial Virulence with RNA

ABSTRACT		
INTRODUCTION		
L. MONOCYTOGENES	Several RNA regulators modulate the saprophytic versus pathogenic lifestyle	- PrfA expression
		- Flagellum biosynthesis: the excludon concept
	Rli55 is a riboswitch-regulated ncRNA that sequesters the two-component response regulator EutV	
	A riboswitch-regulated asRNA controls propanediol utilization	
	Rli27 regulates a cell wall protein important for bacterial survival in blood	
S. TYPHIMURIUM	IsrM regulates the *Salmonella* pathogenicity island 1	
	The leader mRNA of the *mgtCBR* operon senses ATP and proline intracellular levels	
	PinT controls the expression of bacterial virulence genes and causes pervasive changes in host transcripts	
V. CHOLERAE	Qrr 1-5 small ncRNAs regulate colonization and escape from the host	

	TarB controls intestinal colonization	
	VqmR represses a biofilm regulator	
S. AUREUS	RNAIII controls transition from colonization to dissemination mode	
	The *cis*-asRNA $SprA1_{as}$ also controls in trans a hemolytic peptide	
	icaR RBS and 3′-UTR base pairing in the control of biofilm formation	
CONCLUSIONS AND PERSPECTIVES		

Keywords: RNA, virulence, pathogen, regulation

ABSTRACT

Noncoding RNAs (ncRNAs) regulating virulence have been identified in most pathogens. The intent of this review is to discuss RNA-mediated mechanisms exploited by bacterial pathogens to successfully colonize their hosts and infect them. The most representative RNA-mediated regulatory mechanisms employed by two intracellular (*Listeria monocytogenes* and *Salmonella enterica* serovar typhimurium (S. Typhimurium)) as well as two extracellular (*Vibrio cholerae* and *Staphylococcus aureus*) bacterial pathogens are discussed. The RNA-mediated regulators (e.g. thermosensors, riboswitches, *Cis* and *trans* -encoded RNAs) used for adaptation to the specific niches colonized by these bacteria (intestine, blood or the intracellular environment for example) will be reviewed in the framework of the specific pathophysiological aspects of the diseases caused by these microorganisms. A critical discussion of the newest findings in the field of bacterial ncRNAs will be provided showing how examples in model pathogens could pave the way for the discovery of new mechanisms in other medically important bacterial pathogens.

INTRODUCTION

Bacterial pathogens have evolved mechanisms to sense the host environment and to adapt constantly to the specific niche they colonize, regulating exquisitely the production of specialized virulence factors (78). Adaptation of virulence factor production to specific stimuli can be controlled at the transcriptional, post-transcriptional, translational and post-translational levels through complex regulatory networks in bacteria.

More than 40 years ago, the central dogma of molecular biology proposed that DNA contains the information to encode all the proteins of a living organism, while three different categories of RNA translate this code into proteins (22). The three groups of RNA include: messenger RNA (mRNA) also

known as coding-RNA, transfer RNA (tRNA) and finally ribosomal RNA (rRNA). RNA species (Ribozymes) that catalyze biochemical reactions like enzymes do, were then discovered. In addition to tRNA, rRNA and ribozymes, other ncRNAs were identified and they have been found to have complex regulatory roles in mammalian and bacterial cells (26; 100). As a group, these RNAs are termed ncRNAs and they modulate transcription, mRNA stability, translation, and DNA maintenance or silencing through different mechanisms which in bacteria include (34; 65; 85; 96; 103): (i) changes in RNA conformation of the 5' UTR leading to transcriptional read-through/induced premature termination or activation/inhibition of translation (RNA Thermosensors, pHsensors and riboswitches) (41; 56; 61); (ii) perfect base pairing between an antisense RNA (asRNA) and the gene that is encoded on the opposite strand (86); (iii) imperfect base pair interactions of a ncRNA and its target RNA encoded elsewhere on the chromosome (6; 10; 28; 29; 42; 75; 84; 93); (iv) regulation of protein functions by sequestration of proteins from their normal targets or by modification of their enzymatic activity (95; 101); and (v) interactions with DNA (e.g. crRNAs of the immunity CRISPR system) (18; 68). Due to space limitations, the reader is referred to excellent reviews focused on regulatory RNAs and their mechanism of action as well as on the CRISPR/Cas system and the role of crRNAs in virulence (14; 18; 47; 68; 95; 96; 100; 107).

Bacterial ncRNAs size usually varies between 50 and 400 nucleotides (nt) in length (68). Most ncRNAs are transcribed from intergenic regions, while others are transcribed from an mRNA internal promoter. Finally, another class of RNA can be generated from riboswitches and from longer RNAs (16; 17; 43; 56). High-throughput technologies have recently accelerated discoveries in the bacterial RNA field (7). For example, genome wide expression studies using tiling arrays and RNA-seq technologies led to major progress in the discovery of ncRNAs in bacterial pathogens like *L. monocytogenes*, *S. aureus*, S. Typhimurium, *V. Cholerae*, *Helicobacter pylori*, *Chlamydia trachomatis* or *Pseudomonas aeruginosa*

among others (1; 46; 60; 89; 98; 105; 108). The current omics era made possible the use of a 'dual RNA-seq' approach which profiles RNA expression simultaneously in a pathogen and its host during infection being able to reveal the molecular impact of bacterial ncRNAs. This technique is a paradigm to increase the knowledge of the interaction between bacterial pathogens and their hosts, as well as a new tool to discover hidden functions of pathogen genes (105; 106). Finally, a new method termed term-seq was set up to quantitatively map all exposed RNA 3′ ends in individual bacteria as well as in microbiomes. This technique can assess activities of ribo-regulators and allows unbiased genome-wide identification of genes regulated by premature transcription termination (23).

Bacterial ncRNAs modulate the response to environmental changes, control physiology, development and metabolism and regulate virulence in pathogenic bacteria (14; 34; 68; 95; 100). Here we will present some striking examples of ncRNAs in intracellular and extracellular pathogens.

L. MONOCYTOGENES

L. monocytogenes is a gram-positive bacterium that lives in the soil as a saprophyte but is also a pathogen responsible for listeriosis, a food borne disease characterized by bacteremia, meningoencephalitis, abortion or neonatal sepsis and a case-fatality rate of 30% (20). *L. monocytogenes* is able to colonize the intestine, promote its own internalization in mammalian cells, disrupt the internalization vacuole and escape into the cytoplasm, where an actin-based motility system dependent on activation of the Arp2/3 complex by the bacterial surface protein ActA propels the bacterium and allows spreading to neighboring cells (20; 72; 76).

Several RNA regulators modulate the saprophytic versus pathogenic lifestyle.

- PrfA expression

PrfA is a transcriptional regulator belonging to the cAMP receptor protein (Crp)/fumarate nitrate reductase regulator (Fnr) family which regulates most virulence factors of *L. monocytogenes* (internalins InlA and InlB, the pore-forming toxin listeriolysin O, the phospholipases PlcA and PlcB, the Mpl protease, the actin polymerizing protein ActA, the secreted protein InlC, and the sugar phosphate permease Hpt). PrfA translation is controlled by a 5'UTR thermosensor that adopts alternative secondary structures depending on the temperature. At temperatures ≤ 30°C this thermoswitch sequesters the *prfA* RBS due to formation of an RNA hairpin, but at 37°C (temperature of mammalian hosts) the thermosensor is destabilized allowing PrfA translation and virulence factor production (Figure 1A) (41).

In addition to its 5'UTR thermosensor, PrfA is regulated in *trans* by two small ncRNA generated from the S-adenosylmethionine (SAM) riboswitches, SreA and SreB (56). SAM binding to these SAM riboswitches causes formation of a structure that terminates transcription, generates a small ncRNA and prevents synthesis of the downstream mRNA. The small ncRNAs SreA and SreB can base-pair with the RBS region of the *prfA* mRNA and block translation initiation. The impact of the SAM riboswitches on PrfA expression highlights the presence of a subtle network linking nutrient availability and virulence in *L. monocytogenes* (56).

- Flagellum biosynthesis: the excludon concept

Synthesis of the flagellum in *L. monocytogenes* is controlled by an excludon. The name excludon was coined to name a locus where an asRNA simultaneously represses expression of the overlapping transcripts and encodes an mRNA for one or several neighboring genes (86; 98). At 30° C, *L. monocytogenes* expresses flagella and exhibits swimming motility in liquid environment or swarming motility on semi-solid surfaces to access nutrient sources. At host temperature (37° C), the repressor

MogR blocks transcription of flagella genes and switches off flagella formation (47). Among the genes repressed by MogR, the *fli* operon (*lmo0675-lmo0689*) is transcribed opposite of the *mogR* operon and encodes proteins required for the synthesis of the flagellum. Two promoters have been identified for *mogR*: a sigma B dependent promoter P1 at 1,697 nt from the *mogR* ATG and a second promoter P2 at 45 nt from the ATG (98). The mRNA from P2 is constitutively expressed. Interestingly, expression from P1 generates a long asRNA (anti0677) which overlaps the first three genes of the *fli* operon (*lmo0675–lmo0676–lmo0677*) that have divergent orientation (Figure 1B). *lmo0676* and *lmo0677* encode FliP and FliQ, respectively, and together with FliR (*lmo0678*) form the flagellum export apparatus. The long asRNA anti0677 negatively regulates the expression of the sense transcript, inhibiting the synthesis of the Lmo0675, FliP and FliQ flagellum export apparatus while concomitantly allows the expression of MogR. During infection at 37° C, two independent mechanisms switch off the flagellum production: 1) an inhibition mediated by the antisense component of anti0677 and 2) a transcriptional repression mediated by the production of MogR (Figure 1B) (86; 98). Importantly, the *mogR- lmo0675–lmo0676–lmo0677* was the first reported excludon.

Rli55 is a riboswitch-regulated ncRNA that sequesters the two-component response regulator EutV.

The ethanolamine utilization operon (*eut*) is important for *L. monocytogenes* pathogenesis in an intravenous mouse infection model (62). *eut* genes are also important for S. Typhimurium after oral infection (97). The enzymes of the ethanolamine utilization pathway use vitamin B12 as a cofactor and pathogens have evolved sophisticated tools to optimize *eut* expression when both vitamin B12 and ethanolamine are present (62). The *eut* operon is transcribed in the presence of ethanolamine and vitamin B12 under the control of the EutVW two-component system, where EutW is a sensor kinase,

and EutV is an antiterminator which regulates the *eut* messenger RNAs through binding dual-hairpin structures that overlap terminators. EutV thus prevents transcription termination (Figure 1C). *eut* expression also depends on a riboswitch that binds vitamin B12 and controls the expression of the ncRNA, Rli55. In the absence of vitamin B12, a full length Rli55 containing an EutV-binding site is transcribed, which sequesters the two-component response regulator EutV and impedes *eut* transcription. When vitamin B12 is present, the Rli55-riboswitch terminates Rli55 transcription upstream of the EutV-binding site. As a consequence, EutV is free to bind the nascent *eut* transcripts and avoid its premature termination (Figure 1C) (62). Interestingly, a similar mechanism has been described in *Enterococcus faecalis*, where a riboswitch-containing the small ncRNA EutX controls *eut* gene expression by sequestration of the EutV response regulator (25).

A riboswitch-regulated asRNA controls propanediol utilization

Propanediol is an important nutrient source for bacterial pathogens during infection (12; 63). *L. monocytogenes* up-regulates the propanediol utilization operon (*pdu*) during the intestinal stage of infection of mice (3). AspocR is a vitamin B12 riboswitch regulated asRNA which is in a convergent orientation to *pocR*, a gene encoding a transcription factor that is expressed in the presence of 1,2-propanediol, and triggers the expression of the *pdu* genes. In absence of vitamin B12, the long form of AspocR is expressed which inhibits *pocR* expression and consequently the activation of the *pdu* genes. Binding of vitamin B12 to the riboswitch terminates the transcription of AspocR prematurely and transcription of *pocR* can proceed (Figure 1D). This elegant system allows that the *pdu* genes are maximally expressed only when 1,2-propanediol and vitamin B12 are present (63).

Rli27 regulates a cell wall protein important for bacterial survival in blood

After crossing the intestinal barrier, *L. monocytogenes* disseminates through the blood to the spleen, liver, brain and placenta. Lmo0514 is a *L. monocytogenes* LPXTG surface protein highly upregulated during the blood stage or growth within eukaryotic cells. Lmo0514 is required for survival in plasma and for virulence in mice (74; 75). Lmo0514 is transcribed from two different promoters, producing transcripts with 5'UTR of 28 and 234 nt. The 5'UTRs of *lmo0514* transcripts occlude the RBS preventing translation. The transcript containing the long 5'UTR is upregulated in intracellular bacteria and is the target of the trans-acting small ncRNA Rli27, which is also upregulated in the intracellular environment and in blood (75; 77; 98). Rli27 interaction with the 5'UTR of *lmo0514* unmasks the RBS and promotes translation (Figure 1E). This case is another example of a bacterial process related to virulence controlled by different layers of regulation: Firstly, a transcriptional regulation by two different *lmo0514* promoters, and secondly a translational regulation via Rli27.

S. TYPHIMURIUM

S. Typhimurium is a gram-negative bacterium that can infect a broad range of hosts (e.g. humans, cattle, swine and poultry). *Salmonellae* are typically acquired by the oral ingestion of contaminated food or water and cause diverse diseases ranging from self-limiting gastroenteritis to life-threating bacteremia and systemic infection mainly in immunosuppressed hosts, in very young and older individuals (45). S. Typhimurium can invade and survive inside several mammalian cell types. Adaptation to an intracellular lifestyle is controlled by regulatory systems, including two-component systems like PhoP–PhoQ. After internalization S. Typhimurium resides in a specialized phagosomal compartment named the *Salmonella* containing vacuole (SCV), which is mildly acidic and contains low levels of available proline (45; 48; 57).

IsrM regulates the *Salmonella* pathogenicity island 1

The *Salmonella* pathogenicity island 1 (SPI-1) encodes a type III secretion system that forms a syringe-like organelle able to inject effector proteins into the cytosol of eukaryotic cells to manipulate the host cellular functions. SPI-1 is necessary for the initial interaction of *Salmonella* with intestinal epithelial cells (30). IsrM is a small ncRNA encoded in a different pathogenicity island of *Salmonella*. IsrM inhibits *in trans* HilE expression by binding to its mRNA around the RBS sequence, resulting in its translational repression (Figure 2A). HilE is a global negative regulator of the expression of numerous SPI-1 genes by sequestering HilD, a major SPI-1 transcriptional activator (33). IsrM is highly expressed during infection, particularly in the ileum. IsrM is dispensable for *Salmonella* growth in vitro but important for invasion of epithelial cells, intracellular replication inside macrophages, and virulence in mice. This example shows how a ncRNA can be a core regulator of virulence factors controlling the intestinal colonization stage of a foodborne bacterial pathogen.

The leader mRNA of the *mgtCBR* operon senses ATP and proline intracellular levels

MgtC is a virulence factor of S. Typhimurium regulated by PhoP-PhoQ and required for intracellular proliferation inside macrophages and for virulence in mice (52). MgtC is an inner membrane protein that promotes pathogenicity by maintaining ATP homeostasis through the inhibition of the bacterium's own F1F0 ATP synthase (51). MgtC is regulated at various levels: transcription initiation, elongation, mRNA stability, translation, and protein degradation (52). *mgtC* is expressed together with two other genes, *mgtB* and *mgtR*, comprising the *mgtCBR* operon. Transcription of the operon generates a 5' UTR of 296 bp. This long leader RNA encodes two short open reading frames (ORFs): *mgtM* and *mgtP*. These short ORFs can respectively sense ATP and $tRNA^{Pro}$ levels and consequently adopt two sets of alternative stem-loop structures. The stem-loop structures formed affect the coupling/uncoupling of transcription of the *mgtCBR* 5'UTR with translation of *mgtM* or *mgtP*, dictating whether transcription can continue or

not into the *mgtCBR* operon. The SCV is mildly acidic, a condition that can generate high ATP levels inside S. Typhimurium during infection. Elevated intracellular ATP levels promote the formation of a stem-loop at MgtM that allows transcription to continue into the downstream *mgtC* gene promoting virulence (Figure 2B) (49). Similarly, the reduced levels of intracellular charged $tRNA^{Pro}$ uncouple transcription and translation at *mgtP* and favor formation of a stem-loop which enhances transcription of the *mgtC* operon. Moreover, this stem-loop releases the RBS and the start codon of the *mgtC* gene (previously occluded), further facilitating *mgtC* translation (50). Interestingly, the low proline / hyperosmotic stress typically found in the SCV decreases proline levels available to charge $tRNA^{Pro}$, finally leading to enhanced *mgtC* operon transcription (48). The *mgtC* operon is thus controlled by a leader mRNA with two attenuators in tandem that respond to ATP and $tRNA^{Pro}$ levels of the SCV.

PinT controls the expression of bacterial virulence genes and causes pervasive changes in host transcripts

Dual RNA-seq of *Salmonella* infected human cells revealed that PinT is a PhoP-dependent ncRNA that is highly activated after infection (105). PinT simultaneously acts on SPI-1 effectors and SPI-2 virulence genes, shaping the transition from invasion to intracellular replication. Firstly, PinT base pairs with the SPI-1 effectors SopE and SopE2 near the start codon which may cause translational repression. Secondly, PinT directly represses the mRNA encoding the protein CRP which through an unknown mechanism regulates SPI-2 activation (105). This riboregulatory activity of PinT has widespread consequences in coding and noncoding transcripts of infected human cells. For example, PinT reduces the activation of the Suppressor of cytokine signaling 3 (SOCS3, a key regulator of JAK-STAT signaling) whose function is to inhibit the phosphorylation of the STAT3 transcription factor. Moreover, PinT activity decreases the mRNA levels of the pro-inflammatory Interleukin 8 in infected cells (105).

This example shows how a small ncRNA can modulate temporally virulence gene expression and manipulate key host cell pathways and promote bacterial replication.

V. CHOLERAE

V. cholerae is a gram negative, motile bacterium that lives in aquatic environments. *V. cholerae* is transmitted to humans by consumption of contaminated water or food and causes the epidemic diarrheal disease cholera, which is endemic in more than 50 countries and also causes large epidemics (2; 36). A master virulence regulator of *V. cholerae*, ToxT, is regulated post-transcriptionally by an RNA thermosensor which allows translation at 37° C (104). Different *V. cholerae* ncRNAs control the expression of ToxT, which directly activates production of virulence genes crucial for infection, including the cholera toxin (CTX) and the toxin-coregulated pilus (TCP) which allows colonization of the host (58).

Qrr 1-5 small ncRNAs regulate colonization and escape from the host

Quorum sensing (QS) is a microbial cell-to-cell communication-based process leading to the coordination of various common bacterial behaviors including virulence factors expression. In the case of *V. Cholerae*, the phosphorylation of LuxO at low cell density (LCD) promotes transcription of five small ncRNAs named Qrr 1-5. These ncRNAs activate the translation of *aphA*, the LCD master regulator and repress the translation of *hapR*, the high cell density (HCD) master regulator (Figure 3A). At HCD, the phosphorylation of LuxO is inhibited and HapR is expressed (80; 87). Thus, at LCD typically found at early stages of infection, AphA activates the ToxT virulence regulon with expression of the TCP and the CTX (91). At HCD found at late stages of infection, HapR represses *aphA* transcription and consequently, the production of TCP and CTX virulence factors, but activates

expression of proteases such as the *hapA* hemaglutinin/protease gene that permit *V. cholerae* exit from the host (Figure 3B) (35; 96; 111). Moreover, Qrr ncRNAs control the Type 6 Secretion Systems (T6SS) at early stages of infection via two mechanisms: 1) they repress translation of *hapR* which decrease expression of the two small T6SS of *V. cholerae*; and 2) they repress T6SS through base pairing to the mRNA encoding the large T6SS of this bacterium. T6SS are important during the infectious cycle of *V. cholerae* since they contribute to phagocytic cells killing and attack of other prokaryotes (87; 88), as well as to escape from the host during late stages of infection (110). These examples show how Qrr small ncRNAs constitute a dynamic system that allows cell-cell communication in *V. cholerae* to synchronize collective behavior.

TarB controls intestinal colonization

TarB is a ncRNA directly controlled by ToxT and upregulated during infection. TarB is encoded on the TCP island together with many virulence-associated genes (24). TarB stabilized by Hfq downregulates the expression of VspR, a transcription factor present in the *Vibrio* 7th pandemic island-1 which represses the expression of several genes in this island, including a dinucleotide cyclase (DNCV). Importantly, c-AMP-GMP produced by DNCV is required for *V. cholerae* downregulation of chemotaxis and for intestinal colonization, a phenotype related to hyperinfectivity (24). TarB together with Qrr 1-5 tightly control the crucial process of *V. cholerae* intestinal colonization.

VqmR represses a biofilm regulator

A biofilm is a three-dimensionally structured, bacterial community embedded in an extracellular polymeric substance. In *V. cholerae*, VpsT is a major transcriptional regulator of genes involved in biofilm formation that triggers the expression of genes required for synthesis of polysaccharide at LCD

(102). Transcription of *vpsT* is itself activated by the biofilm regulator VpsR (94). A Hfq-dependent ncRNA, VqmR, hybridizes with *vpsT* and presumably inhibits ribosome binding resulting in lower protein levels of VpsT. Thus, VpsT expression is regulated transcriptionally by VpsR (38) and translationally by VqmR (70; 94; 102). Interestingly, these examples show how in contrast to other pathogenic bacteria, *V. Cholerae* quorum sensing represses virulence gene expression and biofilm formation (90).

S. AUREUS

S. aureus is an opportunistic gram positive bacterium ubiquitous in the environment and frequently found in the nose, respiratory tract, and on the skin. It is responsible for nosocomial and community-acquired infections with high mortality. *S. aureus* has been classically classified as an extracellular pathogen, however increasing evidence shows that it has the potential to enter and survive within host cells (31).

RNAIII controls transition from colonization to dissemination mode

The AgrC/AgrA two component system senses *S. aureus* density and controls expression of virulence and other accessory genes. This system encodes an autoactivating peptide (AIP) that is sensed by the histidine kinase receptor AgrC which phosphorylates the response regulator AgrA. Distinct from other systems, the effector is a 514 nt RNA named RNAIII (66; 67). RNAIII is a multifunctional RNA that encodes the cytolytic peptide δ–haemolysin and also acts as a RNA regulating the translation and/or the stability of other mRNAs (39). Importantly, most *S. aureus* clinical isolates contain the Agr system (99). Following a quorum-sensing signal, RNAIII regulates the switch from expression of several surface proteins important for adhesion to secretion of toxins. By doing so, RNAIII controls the transition of the

S. aureus population from a defensive mode (colonization) to an offensive mode (spreading) (Figure 4A and 4B) (11). RNAIII binds to Protein A, Coagulase, Sbi and the fibrinogen-binding protein SA1000 (all of them adhesin factors) mRNAs preventing initiation of translation and recruiting the RNase III which rapidly degrades these repressed mRNAs (9; 15; 19; 38). RNAIII also inhibits translation of *rot* (the repressor of toxins), which finally results in transcription of toxins including α-haemolysin, staphylococcal protease and lipase that contribute to tissue invasion (32; 96). Additionally, RNAIII activates translation of the α–haemolysin by interacting with the 5' UTR of *hla* mRNA and liberating the RBS after the resolution of an inhibitory secondary structure (64). Due to the dependence on RNAIII for the synthesis of exotoxins, it has been suggested that the Agr system is critical for full expression of virulence, particularly during acute infection (11).

The *cis*-asRNA SprA1$_{as}$ also controls in trans a hemolytic peptide

SprA1 is a 30-residue toxic peptide that lyses erythrocytes and has antimicrobial activity against other bacteria (including *S. aureus* itself) (82), a common characteristic shared by other families of peptides (53; 71; 73). SprA1 is encoded in a type I toxin-antitoxin (TA) module within a pathogenicity island. The genes encoding the SprA1 toxic peptide and its asRNA SprA1$_{as}$ are located on opposite strands and possess a 3' overlap. Surprisingly, SprA1$_{as}$ also acts in *trans* by means of a domain outside its target complementary sequence. SprA1$_{as}$ base pair with *sprA1* mRNA, occluding translation initiation signals and preventing its translation. To avoid peptide toxicity during *S. aureus* growth *sprA1* and SprA1$_{as}$ are concomitantly expressed. Acidic and oxidative stresses trigger SprA1 expression by reducing SprA1$_{as}$ levels, which leads to suggest that as an altruistic behavior, some *S. aureus* may express SprA1 to facilitate departure from the phagosomes of host immune cells for spreading (83). This example clearly

shows that the demarcation between *cis*- and *trans*-RNAs is sometimes ill defined and suggests that there is a need for mechanistic re-evaluation of asRNAs (82).

icaR RBS and 3′-UTR base pairing in the control of biofilm formation

Biofilm formation is the main cause of implant infections in orthopedics patients (5). The *S. aureus* biofilm synthesis depends on the enzymes encoded by the *icaADBC* operon (21). IcaR is a transcriptional repressor encoded at the *ica* locus that binds to the *icaADBC* promoter and inhibits *icaADBC* expression (40). A UCCCCUG motif located at the 3'-UTR of the *icaR* mRNA binds the RBS region at the 5'-UTR, inhibiting ribosome loading and generating a double-stranded substrate for RNase III which promotes mRNA decay. Importantly, substitution or deletion of the UCCCCUG motif reduced the 3'-UTR/RBS interaction facilitating *icaR* mRNA translation and consequently inhibiting biofilm formation (79). Whether the 5' UTR and the 3' UTR interaction is inter or intramolecular remains to be elucidated. The present example shows how bacterial 3'UTRs provide regulatory elements to modulate gene expression, a field that has been poorly investigated when compared to their eukaryotes counterparts (79).

CONCLUSIONS AND PERSPECTIVES

Despite the continuously growing diversity of reported mechanisms of action for ncRNA regulating virulence, one still expects that new mechanisms playing a crucial role in the infection process will be discovered. Several aspects of known RNA-mediated regulation are still elusive and should also be soon clarified. First it will be important to investigate ncRNA expression at the single cell level, since the "averaged" data obtained when a population of bacteria is used could hide important regulatory responses and mechanisms employed by specific subpopulations of bacterial pathogens (69; 81). Little is

known about how one specific ncRNA regulates simultaneously different target molecules. New technologies such as single-molecule fluorescence in situ hybridization with super-resolution microscopy may enable the in vivo determination of ncRNA target search kinetics (27). This technique could be instrumental in our current understanding of how bacteria prioritize target mRNAs during infection (27).

New approaches should also provide a deeper understanding of the global regulation of the ncRNAomes. For example, the high throughput biochemical profiling approach termed gradient profiling sequencing, as done recently, can help analyzing global RNA landscapes by partitioning transcripts into diverse coding and noncoding clusters based on their shared RNA–protein interactions (92). In *Salmonella* this method has allowed identification of a large class of structured small RNAs that form stable complexes with the conserved RNA-binding protein ProQ (92). This technique will help to describe functional RNA landscapes in interesting organisms that are emerging from microbiome and environmental studies.

Moreover, there is growing interest in the RNA field since it has been shown that bacterial pathogens ncRNAs may act as signaling molecules able to modulate host gene expression programs (8; 54). This is particularly well illustrated in *Caenorhabditis elegans* where the *E. coli* endogenous ncRNAs OxyS and DsrA modify the expression of host genes impairing the chemosensory behavior and decreasing its longevity, respectively (54). More recently, another study showed that uropathogenic *E. coli* releases membrane vesicles (MV) containing mRNAs, rRNA, tRNAs and small ncRNAs. These MV can be delivered into cultured bladder epithelial cells and their RNA transported into the host cell cytoplasm and nucleus (8). Interestingly, the intestinal microbiota interferes with the microRNA (miRNA) response of the host intestinal tissue upon oral bacterial infection (4). This example shows the existence of a miRNA modulation through which the gut microbiota influences host transcriptional programs during

infection. Conversely, miRNA from the host affect bacterial programs (55). In particular, it has been shown that intestinal epithelial cells, Paneth cells and goblet cells release extracellular vesicles containing miRNAs than can enter *E. coli* and *F. nucleatum* regulating bacterial gene transcripts and growth in vitro (55). Therefore, there is a ncRNA cross-talk between bacterial pathogens and the host (26) suggesting that ncRNAs are potential targets to manipulate the gut microbiome or even bacterial infections in other organs.

In addition, a link between antibiotics exposure or resistance and ncRNA expression has been repeatedly reported: studies in S. Typhimurium and *S. aureus* have first shown that some small ncRNAs are overexpressed as a result of antibiotic exposure (37; 109). More recently, it was shown that many antibiotics resistance genes, in both pathogenic bacteria (e.g. *L. monocytogenes*) and in the human microbiome, are regulated via termination-based ribo-regulators that permit read-through when the antibiotic is present (23). Notably, some *E. coli* and S. Typhimurium small ncRNAs were shown to be important for bacterial viability in response to antibiotics (44; 109).

In conclusion, RNAs are very versatile molecules able to modulate bacterial metabolic routes, antimicrobial resistance and virulence. There is a growing interest to use their properties to generate RNA-based therapeutics in the treatment of genetic disorders, cancers, viruses and bacteria (13; 44; 59; 109). As our comprehension of ncRNAs increases, there is little doubt that their therapeutic applications will expand.

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FIGURE LEGENDS

Figure 1. *L. monocytogenes* ncRNA examples. (A) A thermosensor regulates the expression of the master regulator of virulence PrfA (inspired by (41)). At 30°C the 5'UTR of *prfA* forms a closed stem loop that masks the RBS. At host temperature (37°C), an alternative stem loop of the 5'UTR of *prfA* allows ribosome binding and translation. (B) The flagellum biosynthesis excludon (inspired by (86)). The long asRNA anti0677 encodes the motility gene repressor (MogR) and concurrently downregulates the expression of flagellum export apparatus genes (*lmo0675–lmo0676–lmo0677*) when expressed from the P1 promoter. (C) A riboswitch that binds Vitamin B12 controls expression of the small ncRNA Rli55 which in turn controls expression of *eut* genes (inspired by (62)). In presence of ethanolamine and absence of Vitamin B12, the antiterminator EutV is phosphorylated and sequestered by the full-length Rli55, what finally avoids *eut* genes transcription due to termination at the ANTAR elements. In presence of ethanolamine and Vitamin B12, no Rli55 is produced, what liberates the phosphorylated antiterminator EutV. In this situation, EutV dimerize and bind ANTAR element mediating antitermination of the *eut* transcripts. (D) PocR expression is controlled by vitamin B12 via asPocR (inspired from (63)). In presence of propanediol and absence of Vitamin B12 (Left), AspocR is transcribed as a long antisense RNA and inhibits *pocR* expression. In the presence of Vitamin B12 and propanediol, the riboswitch induces transcriptional termination and transcription of *pocR* is allowed. (E) A cell wall protein (Lmo0514) necessary for plasma survival and virulence is regulated by a small ncRNA (inspired from (75)). Rli27 is a small ncRNA highly induced in the intracellular environment and in blood. In BHI broth, *lmo0514* is expressed from the constitutive P2 promoter which produces a transcript not accessible to ribosomes. However in the intracellular environment an alternative mRNA is generated from the promoter P1 producing a longer mRNA with an occluded RBS. Rli27 base pair with the *lmo0514* long 5'UTR and activates its translation.

Figure 2. S. Typhimurium ncRNA examples. (A) Regulation of SPI-1 virulence factor production in the intestine. The S. typhimurium IsrM small ncRNA is highly induced in the intestine. IsrM targets the *hilE* mRNA and avoids its translation, what finally favors SPI-1 effectors transcription. (B) Regulation of the *mgtCBR* virulence operon by its ATP-sensing leader region (inspired by (49)). The *mgtCBR* leader RNA contains the adenine nucleotides-rich *mgtM* which senses ATP levels. Intracellular ATP concentrations affect the coupling/uncoupling of transcription of the *mgtCBR* leader and translation of *mgtM*, which determines the formation of alternative stem-loops which finally control transcription elongation into the *mgtCBR* genes.

Figure 3. The *V. cholerae* Qrr 1-5 small ncRNAs regulate quorum sensing and virulence (inspired by (80; 88)). (A) At low cell density, the two-component histidine kinase LuxPQ phosporilate LuxO, via LuxU, and activate *qrr* gene expression. The Qrr 1-5 small ncRNAs activate the translation of *aphA* mRNA and repress the translation of *hapR* mRNA through an antisense mechanism what finally leads to CTX and TCP production and colonization of the host. (B) At high cell density, autoinducers (yellow) bind to LuxPQ which dephosphorylates LuxO, leading to cessation of *qrr* gene expression and activation of *hapR* translation. In consequence, proteases and T6SS are expressed what favors escape from the host.

Figure 4. The *S. aureus* RNAIII regulates the transition of the *S. aureus* population from a defensive mode (colonization) to an offensive mode (spreading). (A) At low cell density, AgrC is not phosphorylated which prevents RNAIII production. As a consequence, *spa*, *coa*, *sbi and rot* mRNAs (coding for Protein A, Coagulase, Sbi and the Represor of toxins, respectively) are translated favouring adhesion and colonization. (B) At high cell density, *S. aureus* produce an autoinducing peptide that is sensed by the histidine kinase AgrC causing its autophosphorylation. Then, this phosphoryl group is

transferred to the response regulator AgrA which in turn activates transcription of the regulatory RNAIII. This small RNA posttranscriptionally regulates several target mRNAs. On one hand, pairing of RNAIII to *spa*, *coa*, *sbi and rot* prevents binding of ribosomes. On the other hand, RNAIII interaction with *hla* (coding for α-hemolysin) prevents the formation of an inhibitory structure sequestering the SD which finally facilitates translation. Thus, RNAIII finally induces the production of toxins favouring a spreading mode.

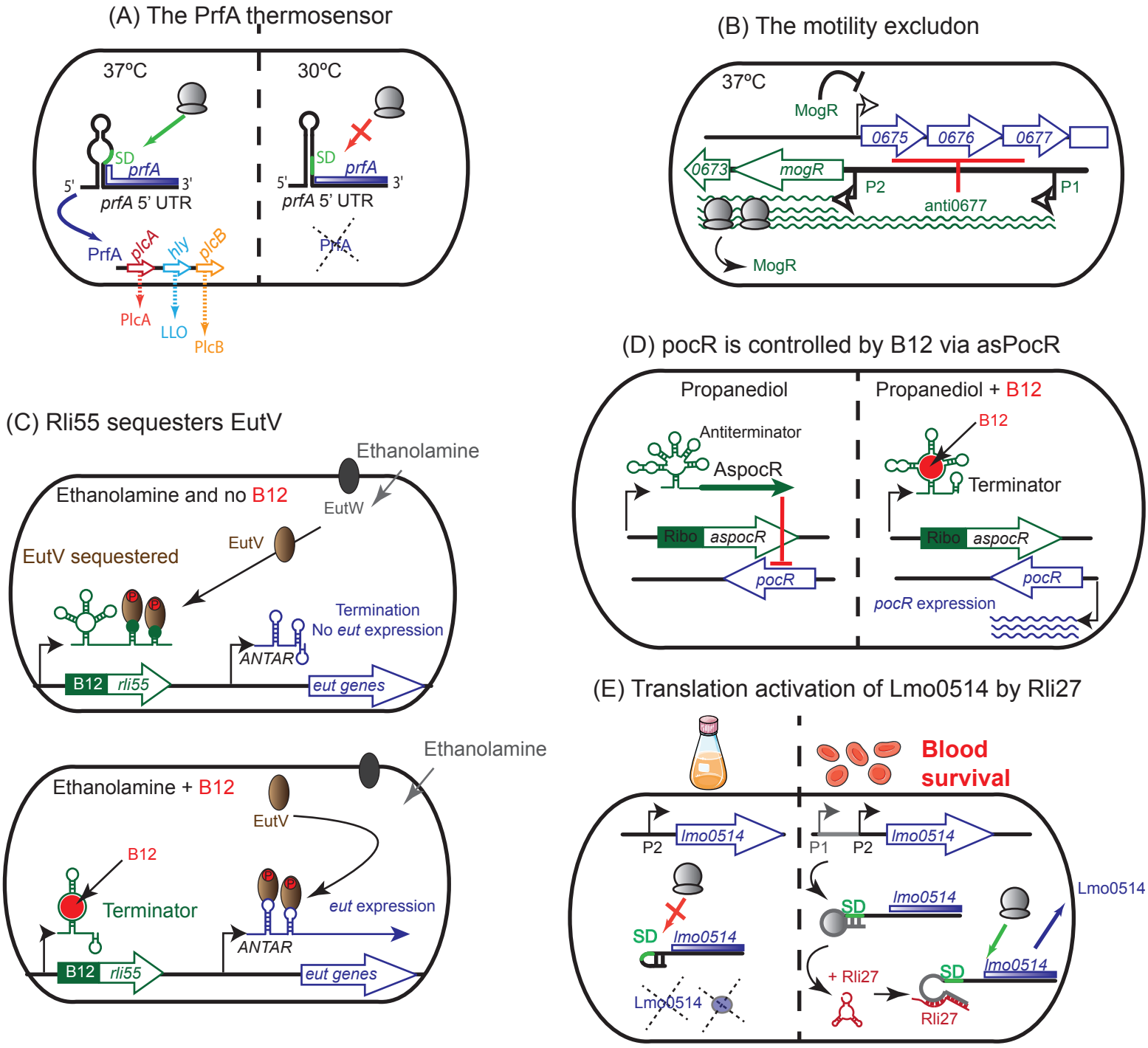
(A) The PrfA thermosensor
37ºC
30ºC
SD
prfA
5'
3'
prfA 5' UTR
PrfA
plcA
hly
plcB
PlcA
LLO
PlcB
(B) The motility excludon
37ºC
MogR
0675
0676
0677
0673
mogR
P2
P1
anti0677
MogR
(C) Rli55 sequesters EutV
Ethanolamine
Ethanolamine and no B12
EutW
EutV
EutV sequestered
Termination
No eut expression
ANTAR
B12
rli55
eut genes
Ethanolamine
Ethanolamine + B12
EutV
B12
Terminator
eut expression
ANTAR
B12
rli55
eut genes
(D) pocR is controlled by B12 via asPocR
Propanediol
Propanediol + B12
Antiterminator
AspocR
B12
Terminator
Ribo
aspocR
pocR
pocR expression
(E) Translation activation of Lmo0514 by Rli27
Blood survival
P2
lmo0514
P1
P2
lmo0514
SD
lmo0514
Lmo0514
SD
lmo0514
Lmo0514
+ Rli27
SD
lmo0514
Rli27

Figure 1

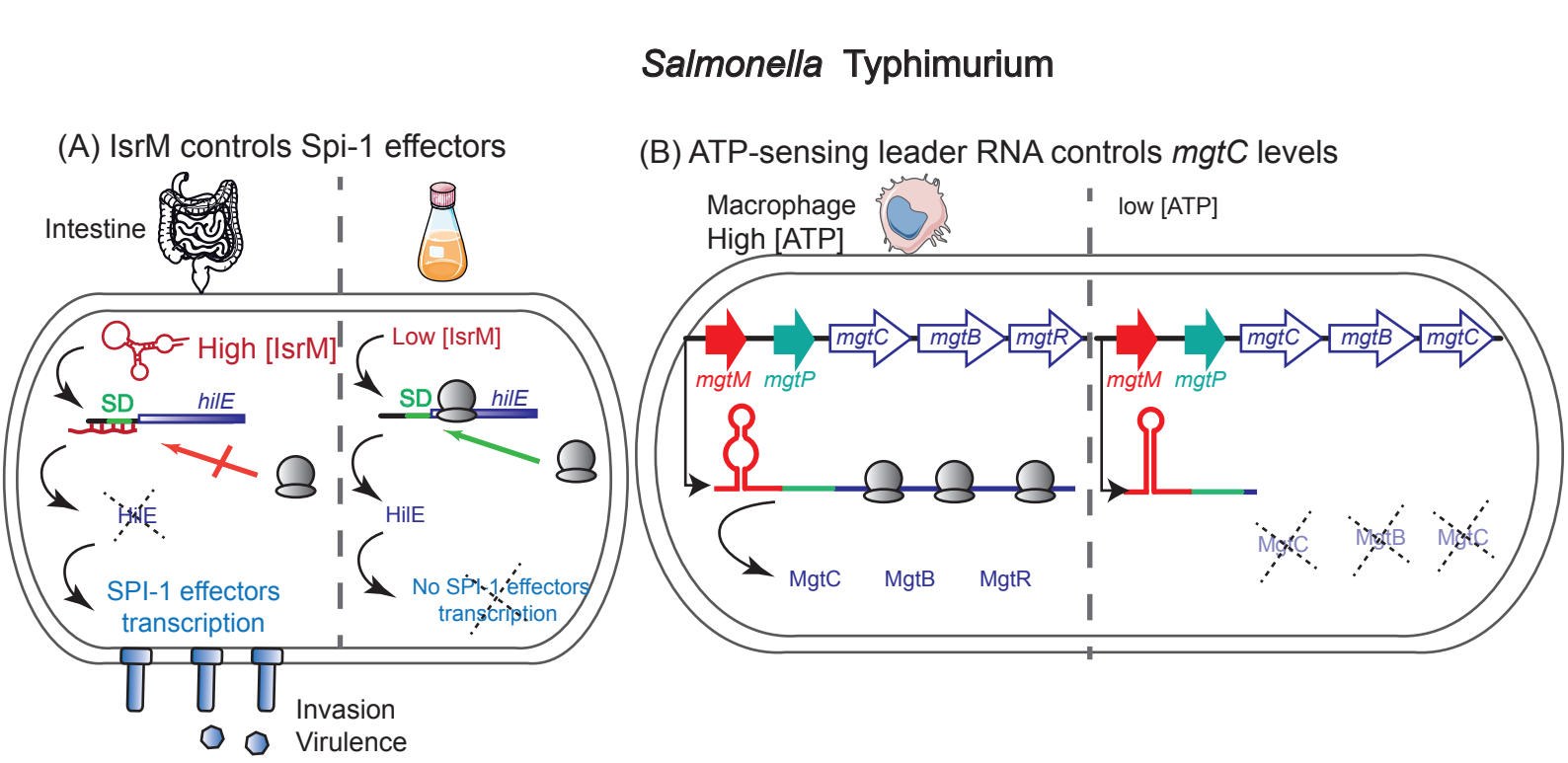
Salmonella Typhimurium
(A) IsrM controls Spi-1 effectors
Intestine
High [IsrM]
Low [IsrM]
SD
hilE
HilE
SPI-1 effectors transcription
No SPI-1 effectors transcription
Invasion
Virulence
(B) ATP-sensing leader RNA controls *mgtC* levels
Macrophage
High [ATP]
low [ATP]
mgtM
mgtP
mgtC
mgtB
mgtR
MgtC
MgtB
MgtR

Figure 2

Vibrio Cholerae

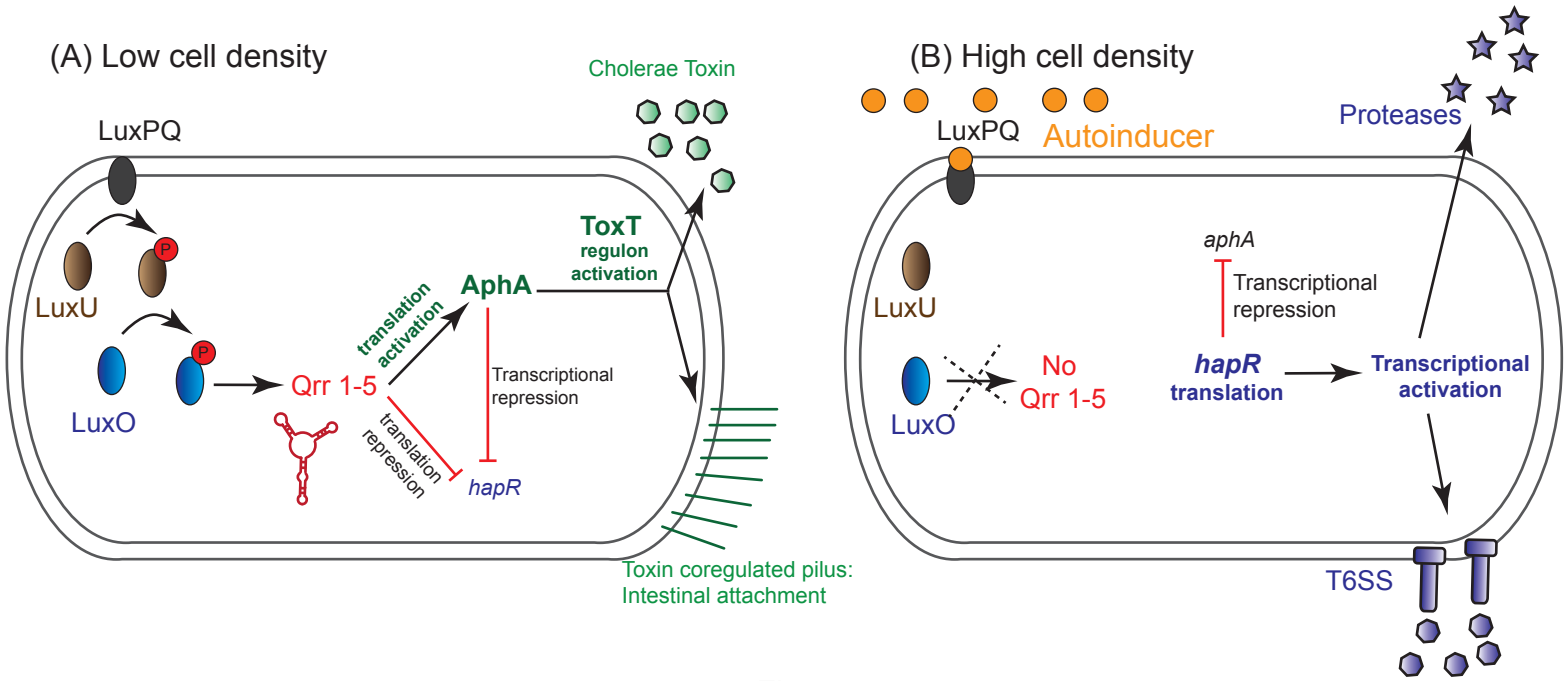

Figure 3

Staphylococcus aureus

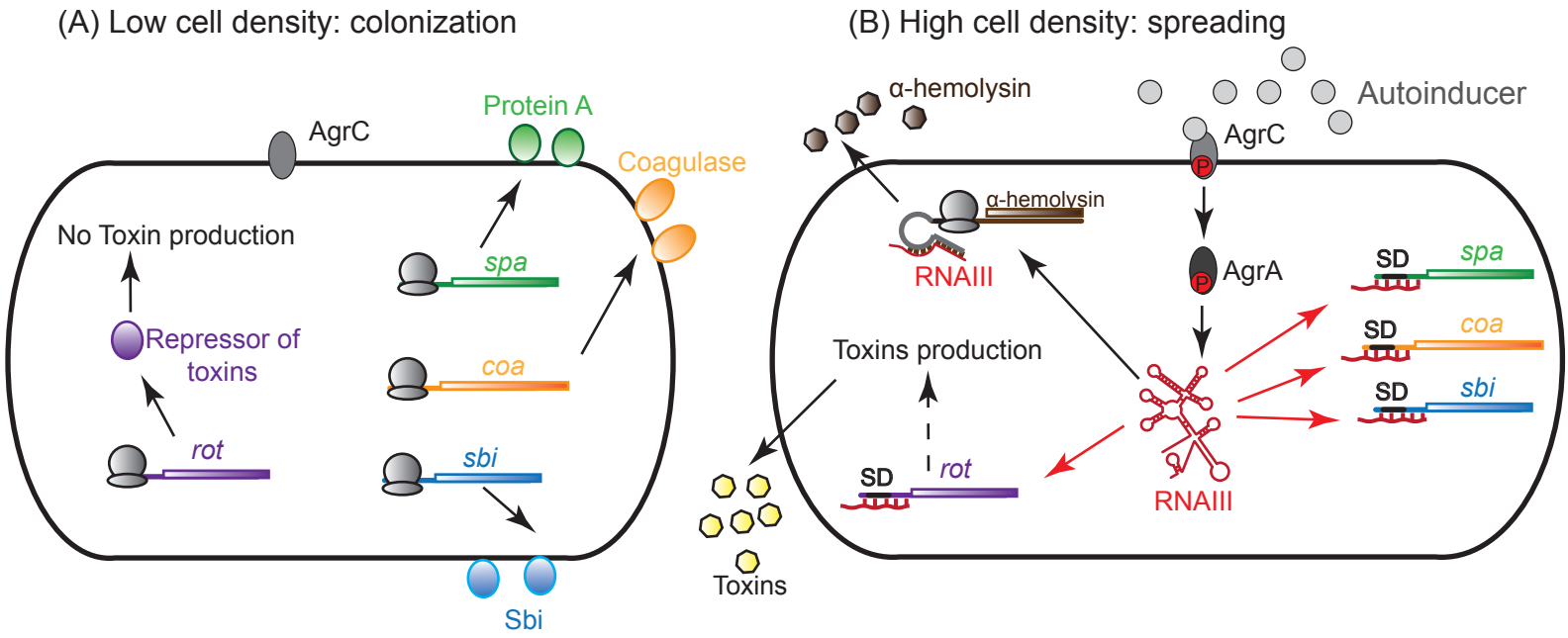

Figure 4