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# Roles of regulatory RNAs in *Vibrio* pathogenic to species of aquaculture interest

Xing Luo

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# Roles of regulatory RNAs in *Vibrio* pathogenic to species of aquaculture interest

Thèse de doctorat de l'Université Paris-Saclay  
préparée à l'Université Paris-Sud

École doctorale n°577 : Structure et Dynamique des  
Systèmes Vivants (SDSV)  
Spécialité de doctorat : Sciences de la vie de la santé

Thèse présentée et soutenue à Orsay, le 09 Septembre 2019, par

**XING LUO**

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# ABBREVIATIONS

ORF	Open Reading Frame
TF	Transcriptional factor
sRNA	small RNA
nt	nucleotides
Co-IP	co-immunoprecipitation
TEX	5' terminator exonuclease
TSS	Transcriptional start sites
RIL-seq	RNA interaction by ligation and sequencing
UTR	Untranslated regions
aa	Amino acids
TCA	Tricarboxylic acid
TCS	Two-component systems
cAMP	Cyclic adenosine monophosphate
CRP	cAMP receptor protein
RBS	Ribosome binding site
asRNA	Antisense sRNA
SD	Shine-Dalgarno
CDS	Coding sequence
TA	Toxin-antitoxin
PSK	Post-segregational killing
RNAP	RNA polymerase
HGT	Horizontal gene transfer
AMP	Antimicrobial peptides
T3SS	Type III secretion system
ECP	Extracellular products
CT	Cholera toxin

TCP	Toxin co-regulated pilus
VPI	Vibrio pathogenicity island
ChIP-Seq	Chromatin immunoprecipitation sequencing
WT	Wide type
OMP	Outer membrane proteins

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# INTRODUCTION

## 1 Regulatory small RNAs in bacteria

Bacteria in various habitats are subject to continuously changing environmental conditions, such as nutrient deficiency, heat and cold stress, oxidative stress, acid stress, cell envelope stress, heavy metal exposure, osmotic stress, and so on. In order to survive, they have to adapt their physiology to these conditions through rapid and possibly dramatic changes in gene expression. In bacteria, gene regulation can be acted at transcriptional, post-transcriptional and translational levels. Transcriptional regulation by a transcriptional factor (TF), a protein that controls the rate of transcription by binding to a specific DNA sequence, is well known (Browning & Busby, 2004). In more recent years, the important roles of regulatory small RNAs (sRNAs) in various cellular processes as major post-transcriptional regulators, especially under specific growth and stress conditions, have been increasingly recognized (De Mets et al, 2019; Durica-Mitic et al, 2018; Dutta & Srivastava, 2018; Frohlich & Gottesman, 2018; Kavita et al, 2018), their studies bringing new insights into gene regulation.

Bacterial sRNAs are mostly noncoding RNAs, 40-500 nucleotides (nt) long and have been found in all eubacteria and archaea where they have been looked for (Wagner & Romby, 2015b). sRNAs can act by either sequestering proteins or base pairing with target mRNAs, affecting mRNA translation or stability (Gottesman & Storz, 2011; Papenfort & Vogel, 2010; Storz et al, 2011; Wagner & Romby, 2015b). Over the past few decades, with more and more bacterial sRNAs being detected and characterized, their intricate and multiple modes of action have become more and more apparent.

### 1.1 sRNAs categories and their regulatory mechanisms

Depending on the mode of regulation, sRNAs can be divided into 3 categories. The first group is mRNA base pairing sRNAs and they can be divided in turn into two sub-categories: *trans*-encoded sRNA and *cis*-encoded antisense sRNA (asRNA). *Trans*-encoded sRNAs are

transcribed from another locus than their target sRNAs while the *cis*-encoded asRNAs are transcribed antisense of their target and hence, perfectly base-pair with their targets.

The second group comprises sRNAs that target proteins. Usually, they interact with the target proteins by mimicking their substrates and modify the activities of proteins. Well studied examples are the 6S RNA (Wassarman, 2018) and CsrB (Vakulskas et al, 2015). The third group comprises *cis*-regulatory RNAs. *Cis*-regulatory RNAs generally correspond to the 5'UTR of the mRNAs they regulate. They control the downstream genes expression by modifying their own conformation, in response to a range of signals, such as various catabolites (riboswitches), temperature (thermoswitches), tRNAs (T-box) and so on (Mellin & Cossart, 2015).

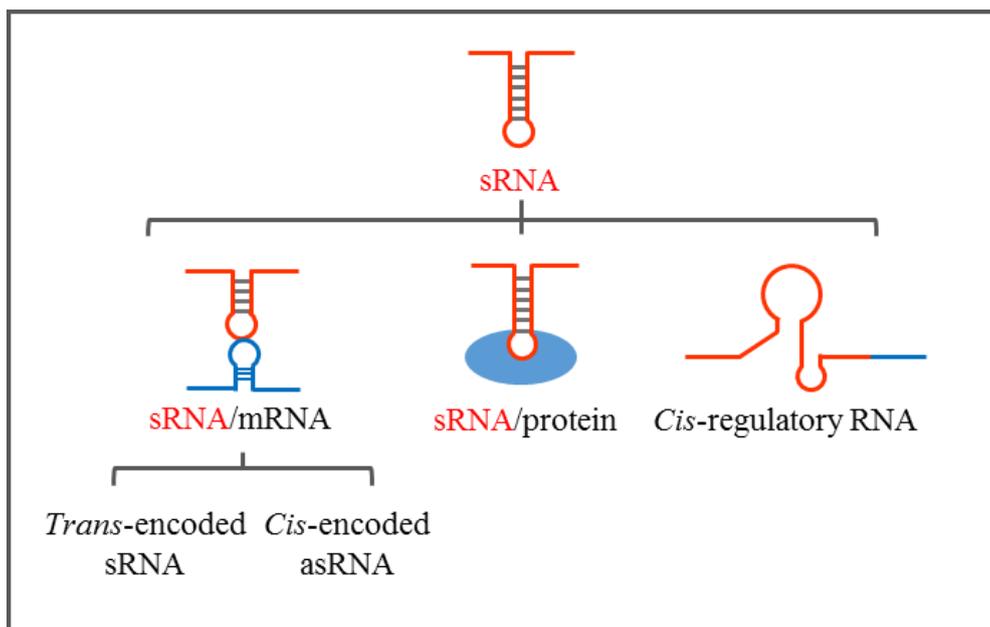


Figure 1 sRNA categories in bacteria

### 1.1.1 sRNAs targeting mRNAs

As mentioned above, the group comprising sRNAs that regulate their target mRNAs in *trans* can be further divided into *trans*-encoded sRNAs and *cis*-encoded asRNA. The differences between these two sub-groups are not only defined by their genetic loci relative to these of their targets, but also by the manners in which they regulate these targets.

### 1.1.1.1 *Trans-encoded sRNAs*

*Trans*-encoded sRNAs are the most extensively studied sRNAs. sRNAs in this class are mostly generated from intergenic regions (i.e. regions separating CDS), which are characterized by the presence of an independent promoter governing their expression and a Rho independent promoter. Because their targets are multiple and located in other regions, they act by short and imperfect base pairing with their targets (Nitzan et al, 2017) and this imperfect pairing often requires an RNA chaperon such as Hfq.

#### *The seed region(s) in sRNAs*

The pairing process is initiated with a fast and high affinity “kiss” through a 6-8 nt seed region of the sRNA often located in the single-stranded region of a stem loop structure at the 5’ end of the sRNA (Gottesman & Storz, 2011; Storz et al, 2011). For example, in *Escherichia coli* the sigma factor  $\sigma^E$  dependent sRNA RybB, whose level increases under conditions causing envelope stress, base pair to and repress the translation of several outer membrane proteins (OMP) mRNAs through its first seven bases (Balbontín et al, 2010).

Although most base-pairing sRNAs are predicted to act via one single-stranded seed region, some sRNAs can have two or even three seed regions, for instance, the widely conserved sRNA GcvB (glycine cleavage system) (Lalaouna et al, 2019). GcvB sRNA is described as a regulator of 1-2% of all mRNAs in *Salmonella Typhimurium* and *E. coli* (Melamed et al, 2016). Up to date, a total of 31 mRNA targets have been validated experimentally, many of which encode amino acid transporters and biosynthesis proteins (Lalaouna et al, 2019). The first seed region R1 is a conserved G/U rich single stranded region and is required for base pairing with C/A rich sequences in the 5’UTR of most of its target mRNAs (Sharma et al, 2007; Sharma et al, 2011). Recently an additional seed region (R3) was shown to regulate the expression of *lrp* (leucine-responsive regulatory protein) (Lee & Gottesman, 2016) and *phoP* (the response regulator of the PhoP/Q two-component systems (TCS)) (Coornaert et al, 2013) and to interact with an sRNA sponge SroC which counteracts GcvB action (Miyakoshi et al, 2015).

Another example of sRNA who has multiple seed regions is Spot 42. Spot 42 was identified

40 years ago in *E.coli* as an RNA that is highly abundant in the presence of glucose (Sahagan & Dahlberg, 1979). Subsequently, people found that cyclic AMP (cAMP) receptor protein (CRP) can directly repress the transcription of Spot 42 (Polayes et al, 1988). Since then, Spot 42 has been demonstrated to directly regulate at least 15 operons involved in multiple aspects of cellular metabolism, including the uptake and catabolism of diverse non-preferred carbon sources (Beisel & Storz, 2011; Papenfort & Vogel, 2011). Indeed, Spot 42 can base-pair its targets via three separated regions (Region I, II and III, shown in figure 3) (Beisel & Storz, 2011).

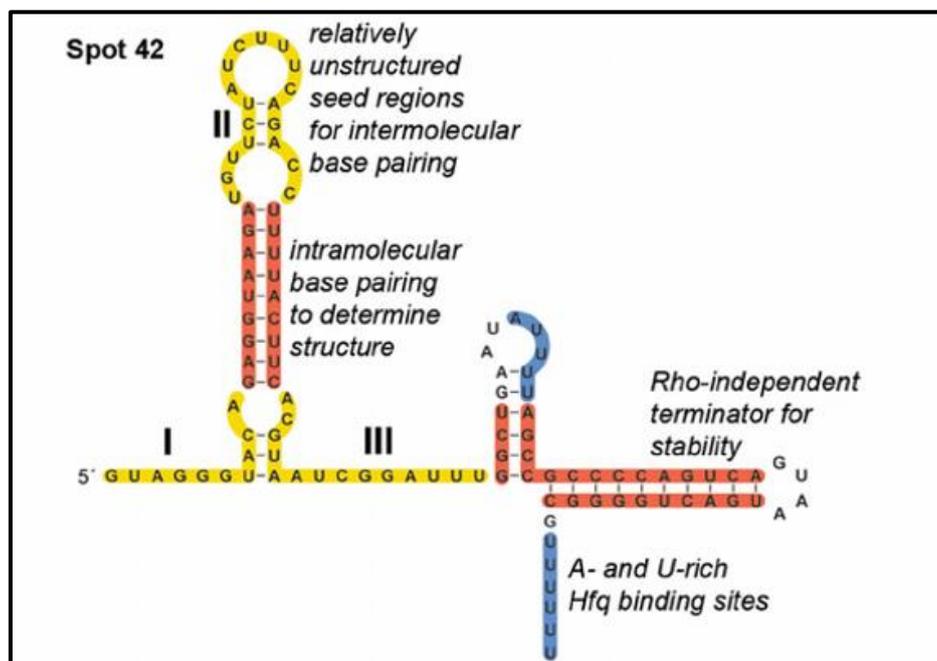


Figure 2 Features of Spot 42 in *E.coli* required for effective regulations (Updegrave et al, 2015).

sRNA genes are evolving rapidly under the selective pressure of different environmental conditions. Therefore, regions of base pairing need to evolve readily in response to physiological constraints (Updegrave et al, 2015). Obviously, with more seed regions, one sRNA can regulate more target mRNAs and play a wider physiological role. Such multi-seed sRNAs may also be more ancestral from an evolutionary viewpoint.

### ***A key factor: Hfq***

For Gram negative bacteria, in many cases the limited base pairing between *trans*-encoded sRNAs and mRNAs need the assistance of an RNA chaperon Hfq (Kavita et al, 2018; Updegrave et al, 2016), a protein identified originally as a **host factor** for RNA phage Q $\beta$  replication (De Fernandez et al, 1968). Nearly half of the sequenced bacterial genomes encode an Hfq homolog (Møller et al, 2002). Series of Hfq co-immunoprecipitation (co-IP) experiments have been conducted in several bacteria leading to the identification of numerous Hfq binding sRNAs (Chao et al, 2012; Christiansen et al, 2006; Dambach et al, 2013; Melamed et al, 2016; Sittka et al, 2008; Updegrave et al, 2016). For example, in *Salmonella*, 32 sRNAs are Hfq dependent, corresponding to half of the characterized sRNAs (Sittka et al, 2008).

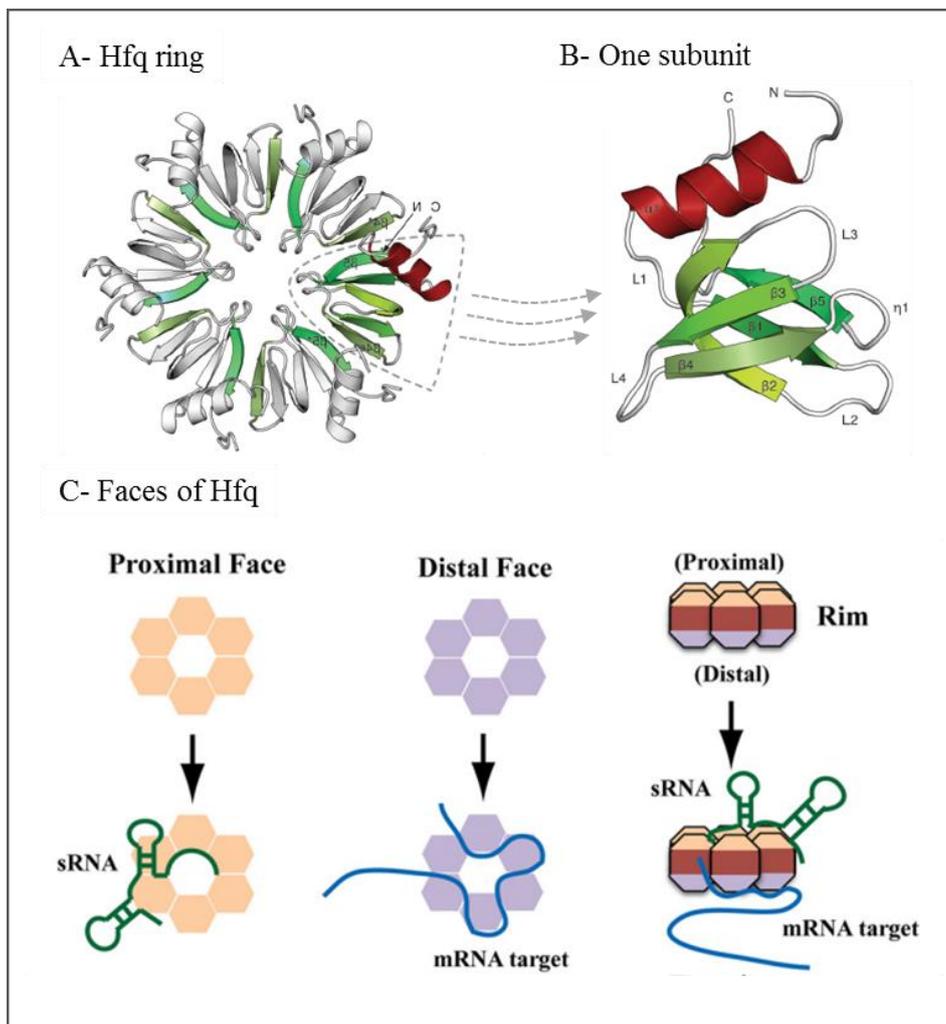


Figure 3 The homo-hexameric structure of Hfq protein (A), subunit (B) and three RNA binding faces of Hfq (C). Hfq protein is composed of 6 subunits. Each subunit contains

an N-terminal  $\alpha$ -helix (red colour) and 5  $\beta$ -strands (green colour) (Sauer, 2013).

The Hfq protein forms a homo-hexameric toroid structure which is assembled from 6 wedge-shaped subunits (Figure 3A) (Sauer, 2013). Each subunit comprises an N-terminal  $\alpha$ -helix, 5 highly twisted and curved antiparallel  $\beta$ -strands and an unstructured C-terminal region (Figure 3B) (Wilusz & Wilusz, 2013). Both sequenced and structural study have shown that the bacterial Hfq protein is a member of Sm or Sm like (Lsm) family of proteins (Wilusz & Wilusz, 2013).

Typically, Hfq participates in sRNA mediated regulation by binding both sRNAs and target mRNAs and stimulating their interaction (Sauer, 2013; Wagner & Romby, 2015b). The Hfq protein can bind to RNAs with 3 faces: the proximal face, the distal face, and the lateral rim (Figure 3C). The proximal face was shown to bind uridine (U) rich or adenosine uridine (AU) rich sequence, mostly in the poly U tail of the Rho-independent terminator of sRNAs (*e.g.* Spot 42 in Figure 2) (Otaka et al, 2011; Sauer & Weichenrieder, 2011; Wilusz & Wilusz, 2013; Zhang et al, 2013). The distal face binds poly A (Wang et al, 2015), (AAN)<sub>n</sub> (Someya et al, 2012) or ARN (R = purine) motif (Horstmann et al, 2012) in the mRNA. Some sRNAs have A-rich sequences that can also bind to the distal face of Hfq (Schu et al, 2015; Updegrove et al, 2016; Wang et al, 2015; Zhang et al, 2013). The lateral rim face contains positively charged arginine patches that can bind in a second AU rich sequence in sRNAs (Dimastrogiovanni et al, 2014; Panja et al, 2013; Sauer et al, 2012; Schu et al, 2015). Depending on the Hfq faces binding to the sRNAs, they can be divided into 2 classes. Class I sRNAs bind to the proximal and lateral rim faces whereas class II sRNAs bind to the proximal and distal faces. Correspondingly, the mRNAs will bind the distal and lateral rim face respectively (Schu et al, 2015).

In addition, Hfq can also act independently as a regulatory RNA binding protein. For example, Hfq binds to the ribosome binding site (RBS) of IS10 transposase mRNA and directly represses translation (Ellis et al, 2015). Also, binding of Hfq protein alone can restructure RNAs, thus affecting RNA expression. Hfq was reported to be able to repress the expression of the mismatch repair gene *mutS* by binding to an (AAN)<sub>3</sub> motif in the 5'UTR of *mutS* mRNA,

rearranging the mRNA secondary structure and modulating the efficiency of mRNA translation (Chen & Gottesman, 2017). Moreover, Hfq binds to and open the structure of the *dgcM* mRNA (which encodes a biofilm formation regulator), thus providing access for the regulatory sRNAs OmrA/B (Hoekzema et al, 2019).

In Gram positive bacteria, the role of Hfq is quite elusive. Hfq is not expressed in some *Staphylococcus aureus* strains (Felden et al, 2011; Geisinger et al, 2006). Even when *hfq* is present, it appears not to be involved in sRNA-mediated regulation (Bohn et al, 2010). Indeed, deletion of the *hfq* gene didn't affect growth in any of the numerous tested conditions (Bohn et al, 2007) and the *S. aureus* Hfq failed to complement *hfq* phenotypes in *Salmonella*  $\Delta hfq$  (Rochat et al, 2012). The situation in *Bacillus subtilis* is similar, the *hfq* mutant having no significant phenotype under 2000 metabolic conditions and chemical treatments (Rochat et al, 2015). The reasons why Hfq is dispensable in some strains but not in others maybe varied. One hypothesis is that extended pairing can overcome the requirement of Hfq, which is mostly the case in *S. aureus* and for the *cis*-encoded asRNAs in *E.coli* (Jousselin et al, 2009). Another hypothesis is that, for those strains who have high GC content, the base pairing between sRNAs and mRNAs can be strong and don't need the aid of Hfq (Gottesman & Storz, 2011).

### ***The regulatory modes of action of trans-encoded sRNAs***

#### **1) Inhibiting the mRNA translation by competing with ribosome loading.**

In bacteria, translation starts with the specific binding of the 30S ribosome subunit to the mRNA at the RBS (Gualerzi et al, 2001). A majority of sRNAs inhibit the mRNA translation by base pairing to a region overlapping or adjacent to the RBS (ranging from 50 nt upstream of the RBS (Sharma et al, 2007) to the fifth codon in the coding sequence (CDS) (Bouvier et al, 2008), thus preventing the loading of ribosome and subsequently leading to the degradation of the untranslated mRNA (Figure 4) (Schmidt et al, 1995; Wagner & Romby, 2015b).

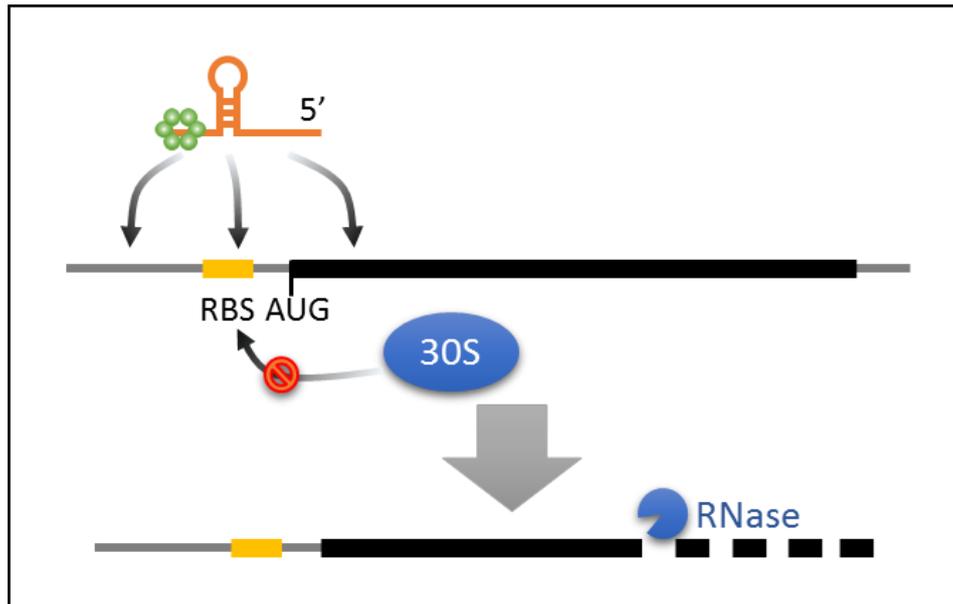


Figure 4 sRNA inhibits translation by competing with ribosome loading and triggering the mRNA degradation.

OmrA and OmrB, two adjacently encoded sRNAs in *E. coli*, contain identical 5' and 3' ends and distinct central regions. Both of them are positively regulated by the EnvZ/OmpR two component system and negatively regulate several mRNAs expression by base pairing with the translation initiation region, such as the OMP-encoding *cirA* and *ompT* messages, their own regulator gene *ompR* (Guillier & Gottesman, 2008), the flagellar regulator *flhD* mRNA (De Lay & Gottesman, 2012) and *csgD* mRNA encoding the transcriptional activator of curli genes (Holmqvist et al, 2010). RsaE, a conserved sRNA in Firmicutes, was found to affect the expression of genes involved in oligopeptide transport (Geissmann et al, 2009), arginine catabolism (Rochat et al, 2018), folate metabolism and the TCA cycle (Bohn et al, 2010) in *S. aureus*. *In vitro* experiments had shown that RsaE binds to the RBS of its target mRNAs to prevent the formation of ribosome initiation complexes (Bohn et al, 2010).

## 2) Activating the mRNA translation by freeing the ribosome binding site.

Under natural conditions, the RBS of some mRNAs is sequestered by intrinsic inhibitory secondary structures. Upon base pairing with the leader sequence of the mRNA, a sRNA can compete with the inhibitory secondary structure and free the RBS. Therefore, the ribosome loads on the RBS and translation is activated (Figure 5) (Frohlich & Vogel, 2009).

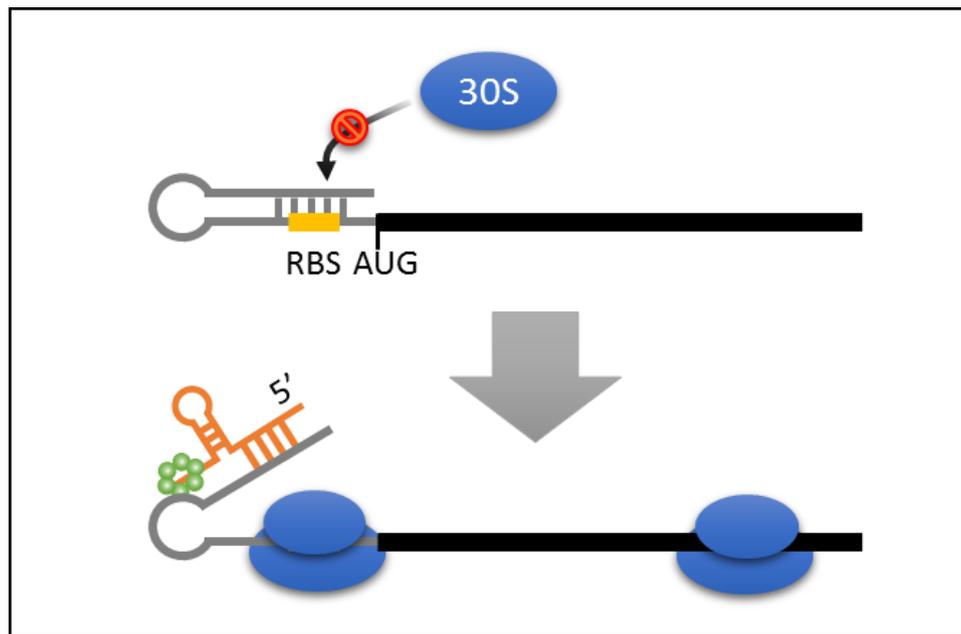


Figure 5 sRNA activates translation by binding to the long 5' UTR of an mRNA and freeing the RBS.

This mode of action of sRNA was first discovered in the human pathogen *S. aureus* during the study of RNAIII. RNAIII is a ~500 nt sRNA, regulated by cell density through quorum sensing and involved in the regulation of many physiological pathways in *S. aureus*, such as stress responses, metabolic adaptation and virulence gene expression (Bronesky et al, 2016). Among its many targets, RNAIII positively regulates the translation of *hla* mRNA, encoding  $\alpha$ -hemolysin, by interacting with the gene 5' UTR and liberating the RBS region (Morfeldt et al, 1995). Other classical cases are the activation of the *rpoS* gene (encoding the stationary phase sigma factor  $\sigma^S$  in Gram-negative bacteria) expression by the sRNAs DsrA, RprA, and ArcZ in response to different conditions (Gottesman & Storz, 2011). DsrA and RprA are induced in response to low temperature (Sledjeski et al, 1996) and cell surface stress (Majdalani et al, 2001) while ArcZ RNA level increases under aerobic condition (Mandin & Gottesman, 2010).

### 3) Recruiting RNases for mRNA degradation.

Bacterial sRNAs can directly trigger mRNA decay, which is mostly initiated by RNase E or

RNase III cleavage (Caron et al, 2010). Once the mRNA is cleaved, it is degraded extremely rapidly (Figure 6). sRNA RyhB is an example of such a sRNA acting through regulating mRNA stability.

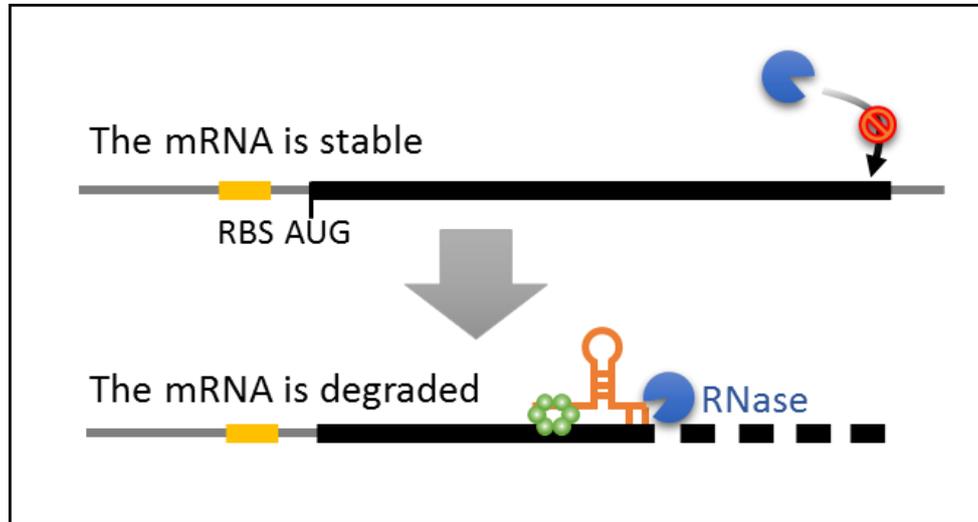


Figure 6 sRNA interaction promotes mRNA degradation.

Iron is an important element needed for survival and growth of bacteria since it is a cofactor of crucial enzymes. Iron homeostasis in bacteria is regulated by the 90 nt long sRNA RyhB and the master regulator Ferric Uptake Repressor Fur (Chareyre & Mandin, 2018). When the environment is iron rich, Fur is activated by iron binding and represses the production of RyhB and iron acquisition proteins whereas it activates the synthesis of iron utilizing proteins (Masse & Gottesman, 2002). When iron is limiting, Fur is inactive, and RyhB accumulates and represses the production of iron utilizing proteins by promoting the degradation of *sodB* mRNA (the iron-containing superoxide dismutase) (Masse et al, 2003), *sdhCDAB* mRNA (succinate dehydrogenase) (Masse & Gottesman, 2002) and the operon *iscRSUA* transcript which encodes the necessary machinery for biosynthesis of Fe-S clusters (Desnoyers et al, 2009). In the latter case, RyhB binds to an internal part of the polycistronic mRNA and promotes the degradation of the 3' part of the mRNA while the 5' part *iscR* mRNA remains stable (Desnoyers et al, 2009).

Another example is *Salmonella* MicC which accumulates during stationary phase and under

conditions of virulence activation. It base-pairs with the 23th-26th codons of the *ompD* mRNA and promotes an RNaseE dependent cleavage without affecting translation initiation (Pfeiffer et al, 2009).

#### 4) Stabilizing target mRNAs

Bacterial sRNAs can also affect mRNA stability to increase expression (Figure 7). *S. aureus* MgrA is a master transcriptional regulator which can negatively affect biofilm formation (Trotonda et al, 2008) and cell autolysis (Ingavale et al, 2005) as well as positively regulate capsule formation (Gupta et al, 2013), the *agr* regulon (Luong et al, 2006) and virulence (Crosby et al, 2016). Recently, it was reported that sRNA RNAIII base-pairs with the 5' UTR of *mgrA* mRNA with two separate domains and significantly stabilizes the mRNA (Gupta et al, 2015). In another example, *S. enterica* sRNA RydC activates the *cfa* mRNA (encoding cyclopropane fatty acid synthase) by binding to the CDS upstream region blocking the RNase E-mediated cleavage in the 5' UTR (Frohlich et al, 2013).

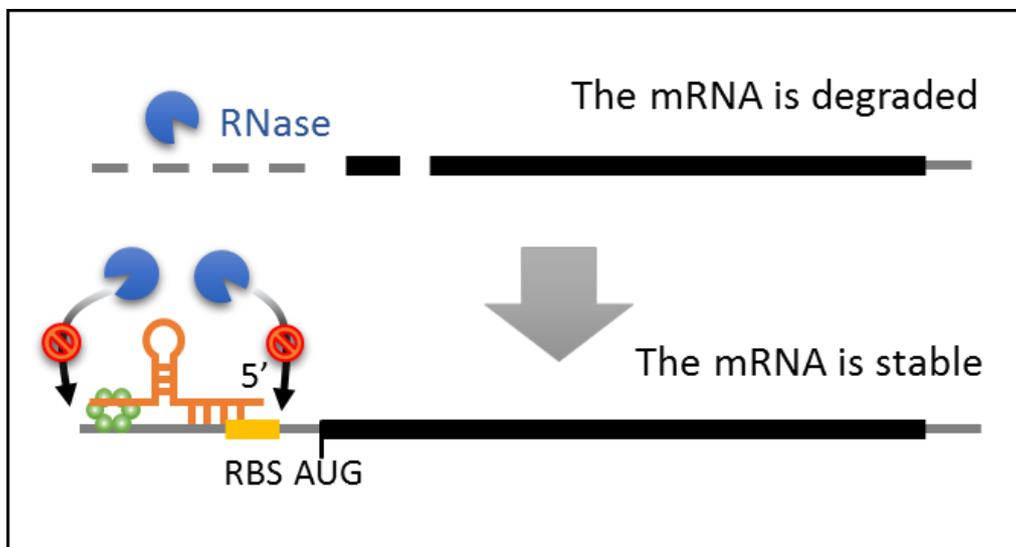


Figure 7 sRNA binds the target mRNA and protects the mRNA from degradation.

#### 5) Affecting Rho-dependent transcription termination

Bacterial sRNAs can not only play a role at the post-transcriptional level but can also act at the transcriptional level by modulating the transcription termination factor Rho activity (Kavita

et al, 2018). Typically, Rho loads RNA at the level of a *rut* (Rho utilization site) site, moves along the RNA until it reaches the elongating RNA polymerase (RNAP) and dissociates the RNA from template DNA (Mitra et al, 2017).

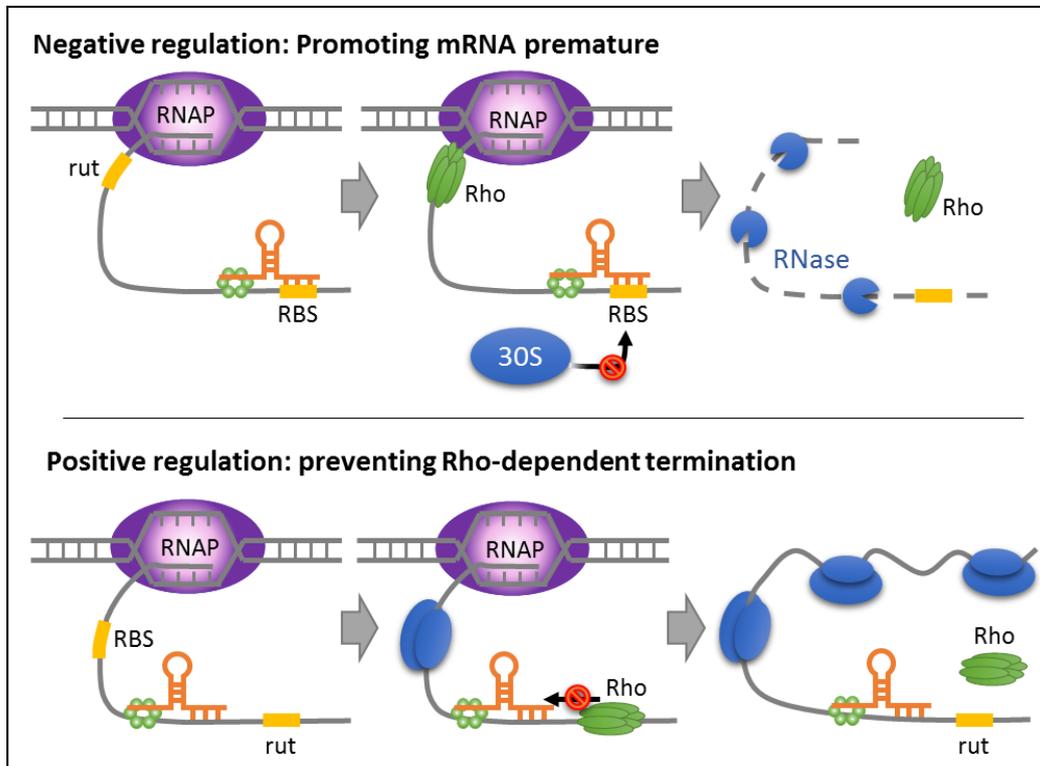


Figure 8 sRNA mediates the Rho-dependent transcription termination.

Up-sRNA base-pairs to the mRNA RBS and blocks translation initiation, making the normally hidden *rut* site become accessible to Rho, thus promoting mRNA transcription termination within the open reading frame (ORF) (Bossi et al, 2012);

Bottom-The interaction between the sRNA and mRNA blocks the loading or translocation of Rho along the RNA, preventing Rho-dependent termination (Sedlyarova et al, 2016).

Usually, the *rut* sites located within the coding sequence are hidden by the translating ribosome. However, once the translation is blocked, those *rut* sites are exposed and become available for Rho binding (Figure 8 up). In *salmonella* and *E. coli*, the sRNA ChiX blocks ribosome loading on the mRNA of the *chiPQ* operon (encoding chitobiose) by base-pairing to the RBS. This allows Rho to bind the *rut* site in the mRNA and cause premature transcription termination of the operon (Bossi et al, 2012).

On the contrary, sRNAs can also positively regulate transcription by inhibiting transcription termination. After annealing with the leader sequences of mRNA, the sRNA blocks the loading or translocation of Rho along the RNA, subsequently preventing the premature Rho dependent termination (Figure 8 bottom). DsrA, RprA, and ArcZ are known to activate the translation of *rpoS* mRNA by base-pairing with the gene 5'UTR, opening an inhibitory secondary structure and allowing ribosome loading (Majdalani et al, 2001; Mandin & Gottesman, 2010; Sledjeski et al, 1996). In addition, these interactions between sRNA and 5'UTR were also found to activate transcription by blocking Rho dependent termination (Sedlyarova et al, 2016). In another interesting example, *Salmonella* sRNA SraL was found to positively regulate the expression of *rho* mRNA by preventing Rho-dependent termination through base-pairing with the 5'UTR. However, the deletion of SraL did not affect the production of Rho as seen by western blot but decreased its activity (Silva et al, 2019).

Furthermore, RNA-Seq and bioinformatics analysis indicated that Rho mediated termination and sRNA mediated anti-termination at the 5' UTR are common in *E. coli* (Sedlyarova et al, 2016).

#### **6) Mimicking another sRNA and regulating its activity**

sRNA can regulate the level of another sRNA by mimicking it, which leads to indirect regulation of the second sRNA's targets.

GlmY and GlmZ are two homologous sRNAs involved in the regulation of the biosynthesis of Glucosamine-6-phosphate (GlcN6P), an essential cell wall precursor (Durica-Mitic et al, 2018). GlmZ is the only positive regulator of GlmS, the GlcN6P synthase. When the level of GlcN6P is high, GlmZ is degraded by RNase E, with the aid of an adaptor protein RapZ. However, when there is a shortage of GlcN6P, the RapZ is sequestered by the highly accumulated GlmY, subsequently, the intact GlmZ promotes the translation of *glmS* mRNA by base pairing with the leader and exposing the hidden RBS (Figure 9) (Gopel et al, 2013; Reichenbach et al, 2008; Urban & Vogel, 2008). Therefore, GlmY and GlmZ operate hierarchically to modulate *glmS* expression, thus mediating the GlcN6P homeostasis.

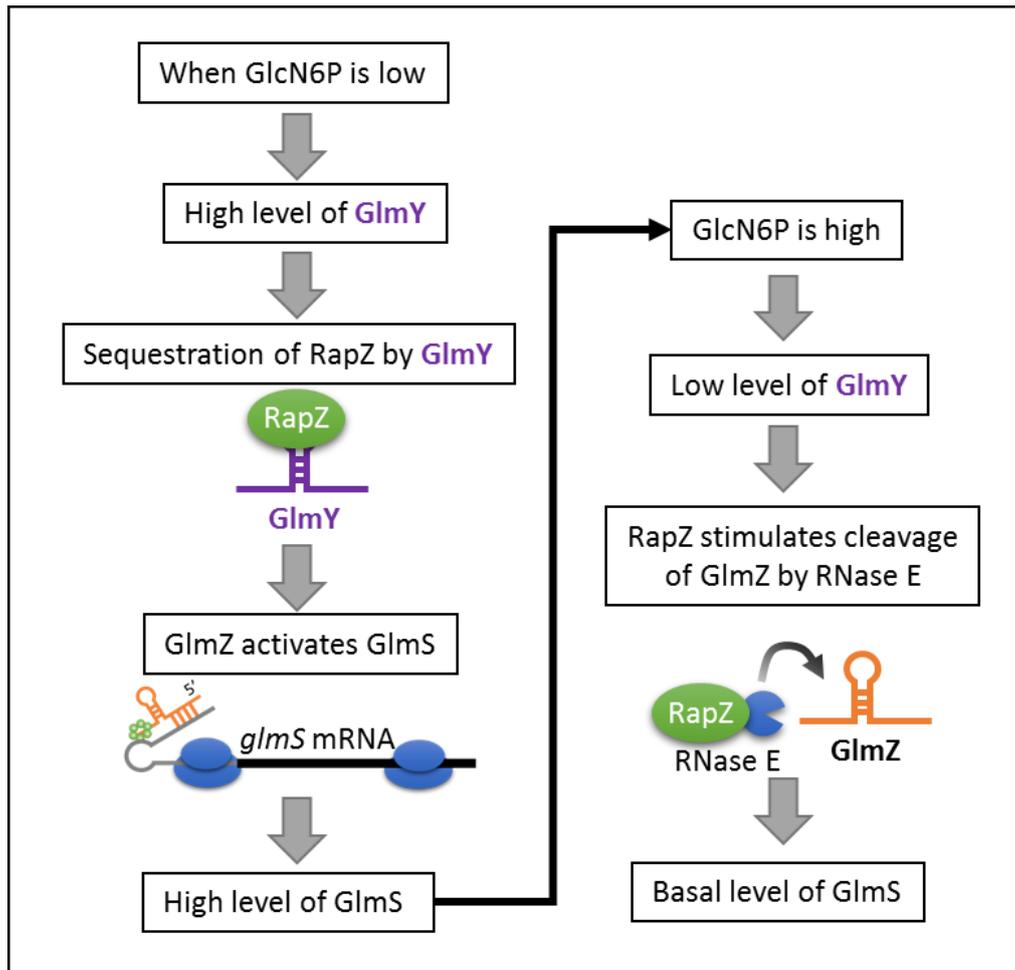


Figure 9 sRNAs GlmY/GlmZ act hierarchically to regulate the GlmS synthesis. The mechanism is explained in the text.

### 1.1.1.2 *Cis-encoded antisense sRNAs*

In contrast to *trans*-encoded sRNAs who are mostly generated from intergenic regions, *cis*-encoded asRNAs are transcribed from the same DNA locus, and from the complementary strand of their target genes. Thereby, asRNAs have complete or extended complementarity to their targets and can form very stable RNA duplex (Azhikina et al, 2015; Georg & Hess, 2011). The first asRNA identified was from the plasmid ColE1 (Tomizawa et al, 1981). The asRNA RNA I controls the initiation of plasmid replication by base pairing to and changing the secondary structure of RNA II, which is itself as a replication primer, priming the synthesis of the leader strand (Itoh & Tomizawa, 1980; Lacatena & Cesareni, 1981). Nowadays, thanks to oriented RNA-Seq and bioinformatics analyses, a plethora of putative asRNA has been identified

in bacterial chromosomes (Bohn et al, 2010; Fozo et al, 2010; Thomason et al, 2015; Toffano-Nioche et al, 2012).

Bacterial asRNAs are similar to *trans*-encoded sRNAs with regard to their regulatory mechanisms. For both types, the RNA hybridizes with its target, but the base pairing is perfect and extended for asRNAs but short and imperfect for *trans*-encoded sRNAs; In both cases, it can cause translation inhibition (Kawano et al, 2007) and/or RNA degradation (Jahn & Brantl, 2013). asRNAs can also modulate the transcription termination by binding to the RNAs, modifying the secondary structure and promoting the formation of a Rho-independent terminator. This is the case of asRNA RnaG which controls the expression of *Shigella flexneri* virulence gene *icsA*, encoding an OMP critical for host colonization (Giangrossi et al, 2010).

In bacteria, asRNAs also act as antitoxins in the type I toxin-antitoxin (TA) system, playing an important role in repressing the synthesis of toxin proteins (Masachis & Darfeuille, 2018). Type I TA systems were firstly discovered as plasmid stabilization modules, working through a post-segregational killing (PSK) effect on the host cells (Gerdes et al, 1997), *e.g.*, the *hok/Sok* system (Gerdes et al, 1986). In case of loss of the plasmid, the sRNA is more unstable than the protein toxin, which can accumulate and kill the host cell. Type I TA systems also exist in bacterial chromosomes with unclear biological functions or selective advantages. Several Type I TA systems have been characterized (Masachis & Darfeuille, 2018). SymE is an SOS induced protein which is an endonuclease. Overexpression of SymE significantly reduces colony formation, decreases protein synthesis and the level of several tested RNAs. The translation of *symE* mRNA is inhibited by the asRNA SymR (Kawano et al, 2007). In another example, overexpression of *ZorO* is toxic to *E. coli*, whereas co-expression of the asRNA *OrzO* counteracts this toxicity and rescues the cells, confirming that the *orzO/ZorO* pair is a true type I TA locus (Wen et al, 2014).

### **1.1.2 sRNAs targeting proteins**

mRNAs are not the only targets of bacterial sRNAs, some of them can interact with proteins directly. By mimicking the DNA/RNA substrates of certain proteins, sRNAs in this class titrate

these proteins and modify their activities. *E. coli* 6S RNA and the CsrB family are two well-studied proteins targeting sRNAs.

### 1.1.2.1 6S RNA

*E. coli* 6S RNA was first discovered more than 50 years ago as an abundant and stable RNA (Hindley, 1967a). In 2000, it was found to form a stable complex with the RNAP containing  $\sigma^{70}$ , the “housekeeping” sigma factor (Wassarman & Storz, 2000), leading to a prevalent functional study of 6S RNA. A recent cryo-electron microscopy structural analysis has provided direct visualization of the *E. coli* 6S-RNAP interaction and has shown the high similarity between the structures of 6S RNA and a DNA promoter upon binding of the RNAP (Chen et al, 2017a). It has been demonstrated by probing experiments that bacterial 6S RNA form a conserved double-stranded RNA hairpin structure with a large bubble in the center (Figure 10A), which is used to mimic the open complex in the DNA promoter (Wassarman, 2018; Wassarman & Saecker, 2006).

6S RNA plays a key role in the transcriptional reprogramming upon shifts from exponential to stationary phase. 6S RNA is expressed all along growth but accumulates to high levels during late stationary phase (~10,000 copies per cell), where the majority of  $\sigma^{70}$  is bound by 6S RNA (Wassarman & Storz, 2000). Sequestration of  $\sigma^{70}$  alters the competition between sigma factors, resulting in an enhanced  $\sigma^S$  activity, aiding the physiological adaptation to stationary phase. This was confirmed by the up-regulation of  $\sigma^S$  dependent transcription *in vivo* in presence of 6S RNA (Neusser et al, 2010) and the finding of a disadvantage in competitive growth and long-term growth defects in the 6S RNA minus strain (Trotochaud & Wassarman, 2004; Wassarman & Storz, 2000). Remarkably, upon outgrowth from stationary phase, 6S RNA is used as a template by RNAP to generate 14-20 nt RNA product (pRNA), a process referred to as pRNA synthesis (Gildehaus et al, 2007; Wassarman & Saecker, 2006). Subsequently, the pRNA-6S RNA hybrid triggers the release of RNAP and is degraded rapidly in the cell (Figure 10 B) (Cavanagh et al, 2012; Wassarman & Saecker, 2006). Therefore, 6S RNA is negatively regulated by a self-encoded RNA upon special environmental conditions.

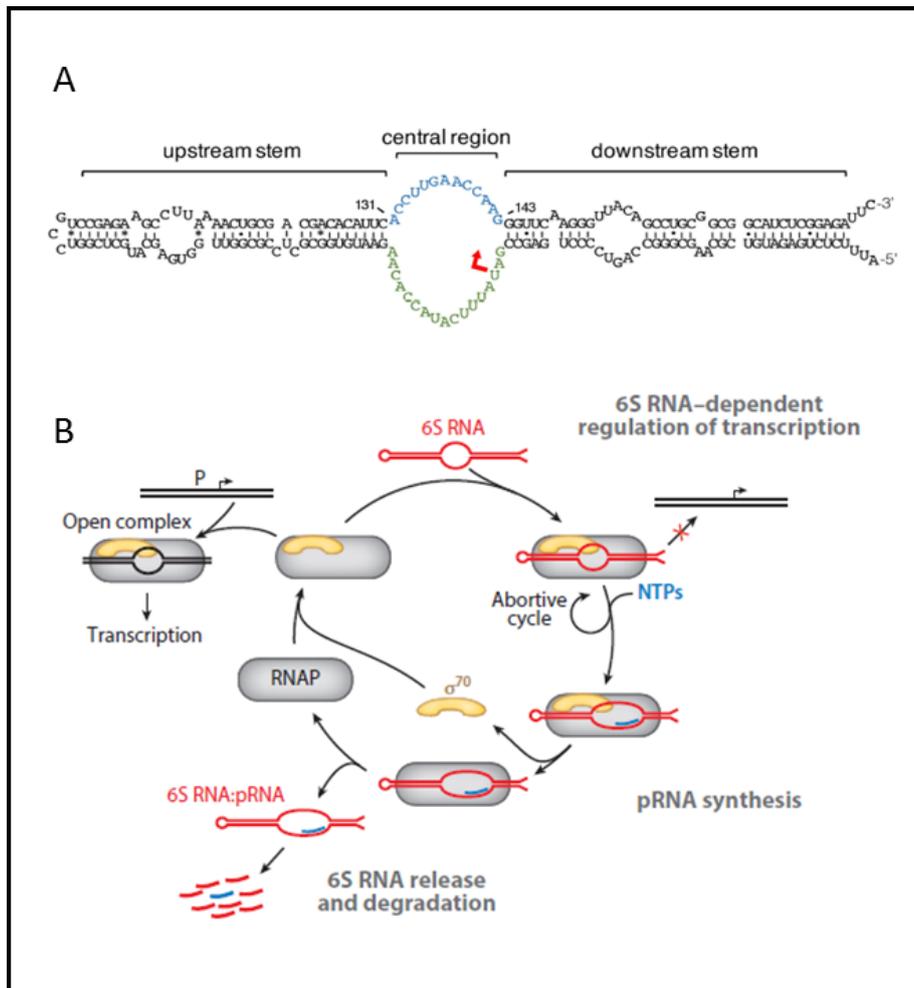


Figure 10 Predicted secondary structure of the *E. coli* 6S RNA (Wassarman, 2018). (A). The upstream stem, central region, and downstream stem in the secondary structure of *E. coli* 6S RNA are indicated. The template central region is in green; the site of initiation for pRNA synthesis is indicated by a red arrow; the non-template central region is in blue. (B) Model for the regulation of 6S RNA function and RNAP availability by pRNA synthesis (Cavanagh & Wassarman, 2014). The mechanism is explained in the text.

6S RNA has been identified in many other bacteria. Although all 6S RNA share low similarity in primary sequence, they have similar secondary structures and the ability to bind to RNAP (Cavanagh & Wassarman, 2014; Wassarman, 2018). 6S RNA in *Synechocystis* sp. strain PCC 6803 has been suggested to contribute to light stress as well as recovery from nitrogen depletion (Heilmann et al, 2017). *Salmonella enterica* serovar Typhimurium 6S RNA accumulates at low

pH, resulting in altered invasion and stress survival (Ren et al, 2017). Some species have multiple 6S RNAs, for example, there are two 6S RNAs in *B. subtilis*, namely Bs6S-1 and Bs6S-2 (Barrick et al, 2005; Trotochaud & Wassarman, 2005). However, only Bs6S-1 RNA affects the timing of sporulation, suggesting specific functions for these two 6S RNA (Burenina et al, 2014; Cavanagh & Wassarman, 2013).

### **1.1.2.2 CsrB family of RNAs**

CsrB and its homologs (CsrC and RsmY) are special sRNAs that can sequester the global regulator CsrA (RsmE) protein and titrate its activity (Vakulskas et al, 2015).

CsrA is a widely conserved RNA binding protein involved in the regulation of carbon metabolism, biofilm formation, motility, virulence, quorum sensing, etc. (Ancona et al, 2016; Babitzke & Romeo, 2007; Muller et al, 2019; Potts et al, 2019; Romeo et al, 2013). In many cases, CsrA regulates gene expression by binding to the mRNA sequences containing GGA motifs in their 5' UTR or in the proximal coding region, affecting translation initiation (Figure 11) (Baker et al, 2002; Dubey et al, 2003; Park et al, 2017), transcription elongation (Figueroa-Bossi et al, 2014), RNA stability (Wang et al, 2005; Yakhnin et al, 2013) or RNA secondary structure (Patterson-Fortin et al, 2013).

CsrB family of sRNAs, whose secondary structure contains many CsrA binding sites (AGGA/ARGGA [where "R" stands for T/C/G]) in single-stranded loops, are the major regulator of CsrA (Vakulskas et al, 2015). One CsrB can bind and titrate several CsrA proteins, therefore competing with the CsrA targets (Figure 11) (Liu et al, 1997). Expression of CsrB/C in *E. coli* is activated by the TCS BarA/UvrY in response to the accumulation of short chain carboxylate compounds such as acetate and formate (Zere et al, 2015). Besides, under preferred carbon source (*e.g.* glucose) conditions, unphosphorylated EIIA<sup>Glc</sup> binds specifically to CsrD, a membrane bound protein, which stimulates the cleavage of CsrB/C by RNase E and PNPase (Figure 11) (Leng et al, 2016; Potts et al, 2018; Suzuki et al, 2006), indicating an increasing activity of CsrA in this condition.

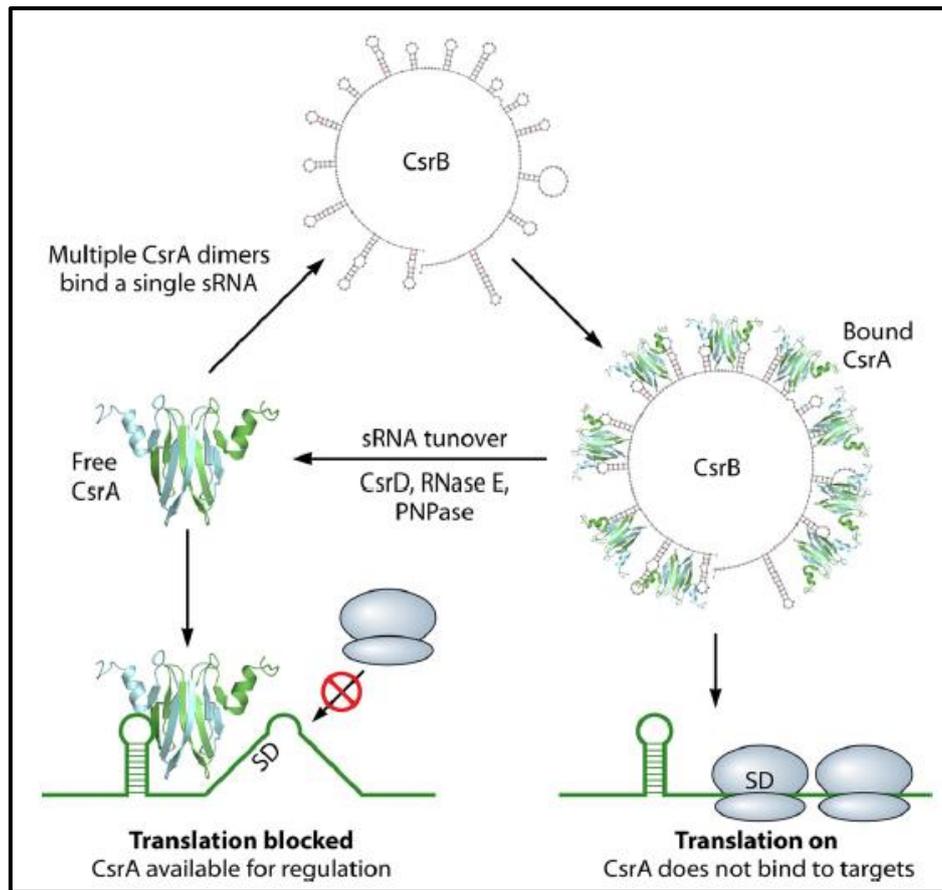


Figure 11 Outline of the Csr system in *E. coli* (Vakulskas et al, 2015). The mechanism is explained in the text.

Csr systems have been identified in a wide range of bacteria, most of them having multiple CsrB sRNAs (Babitzke & Romeo, 2007; Romeo et al, 2013). In oyster pathogen *Vibrio tasmaniensis* (previously named *V. splendidus*) LGP32, 4 CsrB sRNAs have been identified (Nguyen et al, 2018a). These 4 CsrB sRNAs (CsrB<sub>1-4</sub>) are differentially regulated. Indeed, CsrB<sub>2-4</sub> are controlled by the TCS VarS/VarA, orthologs of *E. coli* BarA/UvrY, whereas CsrB<sub>1</sub> is expressed in a VarS/VarA independent way (Nguyen et al, 2018a). VarS is predicted to be a sensor kinase that phosphorylates and activates its cognate response regulator VarA in response to an unknown signal. The VarS/VarA/CsrB system controls the production of secreted proteases (Vsm and PrtV) and  $\sigma^S$ . What's more, a compensatory regulation for the loss of other CsrB copies was evidenced, that is the expression of the remaining *csrB* genes increases when one or several copies are deleted, resulting in the maintenance of a minimal CsrB level (Nguyen et al, 2018a).

Therefore, in *V. tasmaniensis* LGP32, multiple copies of CsrB through differential regulation, allow the bacteria to sense different signals and warrant a high level of functional redundancy. In human pathogen *V. cholerae*, the TCS VarS/VarA regulates the expression of three CsrB sRNAs (CsrBCD). In turns, CsrBCD regulate the expression of ToxR (encodes a major virulence gene regulator) via binding and sequestering CsrA, showing that VarS/VarA/CsrB are critical for the virulence of *V. cholerae* (Mey et al, 2015).

### **1.1.3 Cis-regulatory RNAs**

*Cis*-regulatory RNAs are structured RNA elements located in the long 5' UTR of mRNAs, regulating the downstream gene expression by mRNA conformation modification upon binding small molecule ligands such as metabolic end products (riboswitches), uncharged tRNA (T-box), metal ions ( $Mg^{2+}$ ,  $Mn^{2+}$  etc.) or upon temperature changes (thermometer) (Breaker, 2018; Kreuzer & Henkin, 2018; Lotz & Sues, 2018; Rossmannith & Narberhaus, 2016).

#### **1.1.3.1 Riboswitches**

Riboswitches consist of two domains, the aptamer domain which is used to recognize and bind ligands and the expression platform which regulates the downstream mRNA expression by structural changes (Figure 12) (McCown et al, 2017; Serganov & Nudler, 2013). To date, around 40 distinct classes of riboswitches have been experimentally validated (Breaker, 2018; Lotz & Sues, 2018).

The most widespread mechanisms of gene regulation by riboswitches are transcription termination or translation inhibition by RBS occlusion upon ligand binding, turning off gene expression (McCown et al, 2017). One example is the widely distributed flavin mononucleotide (FMN) riboswitch (Gusarov et al, 1997). In the absence of FMN, the leader region of the *ribD* mRNA, which encodes the riboflavin biosynthesis protein involved in FMN synthesis, folds into a transcriptional anti-termination structure, leading to gene expression. In the presence of FMN, the binding of FMN to the aptamer domain disrupts the anti-terminator and facilitates

the formation of a terminator that shuts off riboflavin biosynthesis (Winkler et al, 2002).

Ligand binding to a riboswitch can also lead to gene activation by transcription anti-termination or translation activation (Lemay et al, 2011). *ykkC* motif RNAs was the longest orphan riboswitch candidates and they are widespread in bacteria (Barrick et al, 2004). Recently, Nelson *et al.* found that the predominant subtype of *ykkC* motif RNAs selectively bind free guanidine and regulate genes encoding proteins that overcome guanidine toxicity such as urea carboxylases, and multi-drug efflux pumps. As with many *ykkC* motif RNAs, part of the aptamer region overlaps the downstream terminator: the presence of the guanidine disrupts the formation of the terminator and increases the downstream gene expression (Nelson et al, 2017).

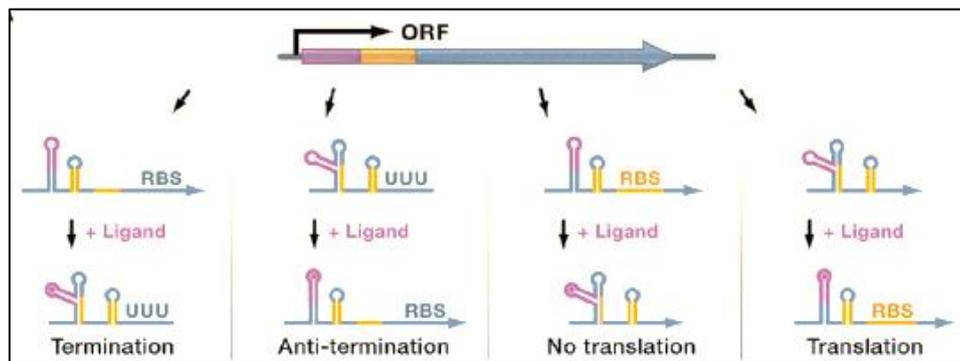


Figure 12 Riboswitches are composed of an aptamer region (pink) and an expression platform (orange) in the 5'UTR of an mRNA (blue).

Left panel- Upon ligand binding, the structure of the aptamer region is modified and the terminator forms, resulting in transcription termination. Middle-left panel- On the contrary, upon binding the ligand, the terminator structure in the aptamer region is destroyed and the anti-terminator forms. Ligand binding can also lead to translation inhibition. Middle-right panel- Upon ligand binding, the exposed RBS becomes unavailable, preventing translation. Right panel- In some cases, the RBS is sequestered by the stem-loop structure in absence of the ligand and becomes accessible in presence of the ligand through secondary structure modification (Lotz & Suess, 2018).

### 1.1.3.2 RNA thermometers

The RNA thermometers (RNAT) are also located in the 5' UTR region of mRNAs, but in

contrast to riboswitch, they regulate the translation of downstream mRNAs in response to temperature changes. Most of the RNATs are heat-inducible: the RBS is sequestered in a hairpin structure at low temperature and exposed upon hairpin destabilization at high temperature (Loh et al, 2018). For example, in *E. coli* the heat shock transcription factor  $\sigma^{32}$  encoded by the *rpoH* gene is induced at high temperature when the mRNA inhibitory secondary structure is open and translation is initiated (Morita et al, 1999).

In the pathogen *L. monocytogenes*, the gene encoding the transcriptional regulator PrfA, an activator of several virulence genes, is also regulated by an RNAT located in the 5'UTR of the mRNA. When the bacteria enters the human body (37°C) from outside (<30°C), the stem-loop structured 5' UTR unfolds, freeing the RBS and activating the translation of PrfA (Johansson et al, 2002).

Also, in this species, Loh *et al.* identified two S-adenosylmethionine (SAM) riboswitches SreA and SreB that can also act as *trans*-acting sRNAs. As other riboswitches, they regulate their downstream gene expression, genes that are involved in methionine or cysteine metabolism. In addition, in nutrient rich medium, when the terminator forms, these two riboswitches can downregulate the expression of the transcriptional regulator PrfA by base pairing with the mRNA in *trans* (Loh et al, 2009). In this case, PrfA is regulated by both riboswitch-derived sRNAs and RNAT.

### 1.1.3.3 Leader peptides

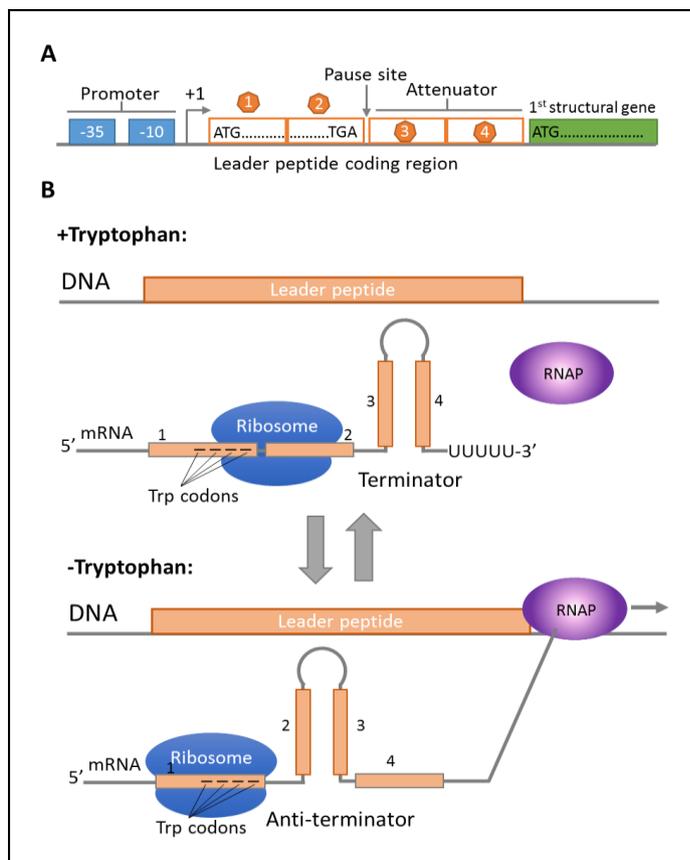


Figure 13 Structure (A) and mechanism (B) of leader peptide regulated transcriptional attenuation of the *E. coli* tryptophan biosynthetic operon. See text for details.

Transcriptional attenuation is an important method used by bacteria to regulate gene expression in response to changes in the environment. Attenuation of many amino acid biosynthetic operons (*e.g. trp, his, leu, ilv, pheA*), particularly in enteric bacteria, are controlled by leader peptide translation (Ames et al, 1983; Carter et al, 1986; Gollnick & Babitzke, 2002; Yanofsky, 2001).

The leader peptide region locates between the transcription start site and the first structural gene of the operon (Figure 13A). It comprises a short polypeptide coding sequence which contains multiple codons for the regulat-

ing amino acid, and a transcription termination site, the attenuator (Figure 13A). The role of this polypeptide coding sequence is to monitor the cellular supply of amino-acylated tRNA(s) for the regulatory codons. For example, the *E. coli* tryptophan biosynthetic operon *trpEDCBA* (Figure 13B) (Salmon et al, 2006b; Yanofsky, 1981). Soon after transcription of the *trp* operon is initiated, the 1:2 stem-loop structure forms in the nascent transcript and causes the transcribing RNAP to pause at the end of stem 2. This allows the initiation of translation of the leader peptide. When the tryptophan is excess in the environment, the level of tRNA<sup>Trp</sup> is high enough to allow the translation of tandem *trp* codons and the ribosome can move to the end of the leader peptide. In this case, the terminator structure (stem-loop 3:4) forms and the expression

of downstream gene is prevented. On the other hand, if the tryptophan is in short supply, the level of tRNA<sup>Trp</sup> is low and translation of leader peptide will be impeded. In this case, the anti-terminator structure (stem-loop 2:3) forms, resulting in transcriptional read through into the *trp* structural genes (Figure 13B).

Interestingly, in *Sinorhizobium meliloti*, under condition of tryptophan sufficiency, the liberated leader peptide RNA rTrpL base-pairs with *trpD* and destabilizes the polycistronic *trpDC* mRNA (another tryptophan biosynthesis operon in this bacteria) (Melior et al, 2019b). So in addition to SreA/B in *L. monocytogenes*, this is another example of multifunctional mRNA leader with regulatory functions in both *cis* and *trans*.

## 1.2 How many sRNAs are there?

One important question about sRNAs is how many are there encoded by a genome? In the early time (before the 2000s) of sRNA exploration, most known sRNAs had been discovered fortuitously (Wassarman et al, 1999). 4.5S RNA (Hindley, 1967b), 6S RNA (Brownlee, 1971), Spot 42 (Ikemura & Dahlberg, 1973), tmRNA and RNase P (Jain et al, 1982) were detected because they are abundant sRNAs. CsrB (Liu et al, 1997; Romeo, 1998) was identified on the occasion of studying its protein target CsrA and OxyS (Altuvia et al, 1997) because it is encoded next to its regulator OxyR. MicF (Andersen et al, 1987), DicF (Faubladier et al, 1990), DsrA (Sledjeski et al, 1996) and RprA (Majdalani et al, 2001) were discovered when certain genomic fragments were overexpressed. More recently, with the growing recognition of the importance of sRNAs in bacterial gene regulation, genome-wide studies have been carried out to identify them in a more systematic way.

### 1.2.1 Approaches to screen novel sRNAs

As shown in Table 1, in *E. coli*, genome-wide searches for novel sRNAs started in 2001 (Argaman et al, 2001; Rivas et al, 2001; Wassarman et al, 2001). At that time, sRNAs were predicted by bioinformatic strategies that focused on the conservation of intergenic regions,

associated with orphan promoters and terminators (Argaman et al, 2001) or RNA secondary structures (Rivas et al, 2001). However, all these strategies have an intrinsic limitation, *i.e.* that sRNAs generated from 5' or 3' UTR of a long mRNA or from the opposite strand of a coding region (asRNA)) could not be thus detected. Shortly after, shot-gun cloning based RNomics approach extended largely the number of sRNA candidates (Kawano et al, 2005; Vogel, 2003). Knowing that a large number of studied sRNAs were reported to be Hfq dependent, Zhang *et al.* (Zhang et al, 2003) used Hfq co-IP in combination with genomic microarrays to identify Hfq dependent sRNAs. As a consequence, 20 new sRNAs were discovered (Zhang et al, 2003). The development of deep sequencing techniques increased the detection sensitivity significantly. In 2015, a differential RNA-Seq (dRNA-seq) approach associated with transcriptional start sites (TSS) mapping, succeeded to identify >5400 potential asRNA in *E. coli* (Thomason et al, 2015). Toffano-Nioche *et al.* developed a computational pipeline (DETR'PROK: detection of ncRNAs in prokaryotes) to process RNA-Seq data to identify ncRNAs and UTRs in *V. splendidus* and *E. coli* (Toffano-Nioche et al, 2013). In 2016, Melamed *et al.* developed a broadly applicable methodology termed RIL-seq (RNA interaction by ligation and sequencing), which integrated experimental and computational tools for *in vivo* transcriptome-wide identification of interactions involving Hfq-associated sRNAs. They uncovered additional Hfq-bound sRNAs together with their *trans* encoded targets (Melamed et al, 2016). Thus far, a number of 80-100 sRNAs was experimentally validated in *E.coli* (Gottesman & Storz, 2011; Huang et al, 2009).

Table 1 The development of genome-wide sRNA identification approaches in *E. coli*

Approaches of genome-wide identification (prediction) of novel sRNAs in <i>E. coli</i>	Output	References
sRNAs were predicted on the basis of intergenic region sequences of 4 related bacterial genomes and conserved RNA secondary structure.	11 sRNAs	(Rivas et al, 2001)
sRNA prediction was based on 1) conserved intergenic regions in 4 closely related bacterial genomes, 2) transcription initiation signal ( $\sigma^{70}$ binding sequence) and termination signal (Rho-independent terminators), 3) 50-400 nt.	23 sRNAs	(Argaman et al, 2001)

sRNA prediction was based on 1) conserved intergenic regions in 4 closely related bacterial genomes, 2) analysis results of transcripts detected by high-density oligonucleotide probe arrays.	23 sRNAs	(Wassarman et al, 2001)
Shot-gun cloning approach (RNomics). Separating and recovering 50-500 nt RNAs from the polyacrylamide gel → Sequencing.	62 sRNAs	(Vogel, 2003)
Co-IP with Hfq → direct detection of the bound RNAs by hybridization on DNA microarrays.	20 new Hfq binding sRNAs	(Zhang et al, 2003)
Separating and recovering 30-65 nt RNAs from the polyacrylamide gel → Sequencing and data analysis.	5' and 3'UTR derived sRNAs and a few asRNAs	(Kawano et al, 2005)
RNA-Seq (Illumina)	10 new sRNAs and 19 regulatory leader sequences	(Raghavan et al, 2011)
Deep sequencing analysis, focusing on RNAs < 200 nt.	229 sRNA candidates	(Shinhara et al, 2011)
Galaxy framework based computational pipeline DETR'PROK (detection of ncRNAs in prokaryotes)	346 sRNA candidates	(Toffano-Nioche et al, 2013)
Hfq co-IP → deep sequencing.	67 asRNAs	(Bilusic et al, 2014)
RNA extraction → 5' terminator exonuclease (TEX) and tobacco acid pyrophosphatase (TAP) treatment → deep sequencing → transcriptional start site mapping → expression analysis	>5400 potential asRNA loci. 14 were confirmed by northern blot	(Thomason et al, 2015)
RIL-Seq (RNA Interaction by Ligation and Sequencing) <i>hfq</i> tagged <i>E. coli</i> → In vivo protein-RNA crosslinking → coIP with Hfq and bound RNAs → RNA ligation → RNA-Seq	1/3 novel sRNAs were identified. New targets for known sRNAs.	(Melamed et al, 2016)

## 1.2.2 Challenges to screen sRNAs

It is however difficult to tell the true number of sRNAs in bacteria because there are still some difficulties to identify all of them, even with the approaches mentioned above.

Firstly, some sRNAs might be missed if the bacteria were not grown under specific conditions (Gottesman & Storz, 2011; Wagner & Romby, 2015b). For examples, DsrA accumulates at low temperature (Sledjeski et al, 1996), RprA is induced by cell surface stress (Majdalani et al, 2001) and ArcZ RNA level increased under aerobic condition (Mandin & Gottesman, 2010). To decrease the biases created by the analysis of only one growth condition, it is better to integrate data from different conditions when screening sRNAs.

Secondly, although a majority of sRNAs are transcribed from intergenic regions (Figure 14A), a little of them were found to be generated from the 5' or 3' UTR of long mRNAs or

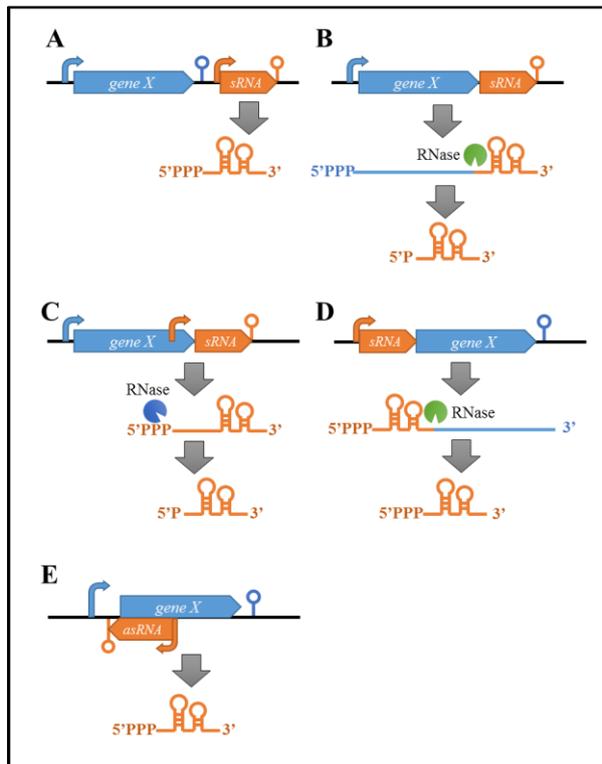


Figure 14 sRNA candidates are encoded within various genomic elements. sRNAs can be generated from the IG region (A), an opposite strand of another gene (E), or 5'UTR (D) or 3'UTR (B and C) of a long mRNA by processing.

expressed from the opposite strand of other genes (Kawano et al, 2005) (Figure 14BCDE), which makes it more difficult to identify them by transcriptomic analysis. In *S. aureus*, Teg49, an sRNA derived from the 5' UTR of the *sarA* mRNA, encoding a transcriptional regulator of several virulence genes, autolysis, biofilm formation, *etc.*, was reported to affect the virulence in *S. aureus* independently of SarA (Kim et al, 2014). Recently, the RNA-Seq analysis of a *teg49* mutant revealed that 34 putative virulence genes and a number of metabolic genes are specifically regulated by this sRNA (Manna et al, 2018a). CpxQ is an sRNA processed from the 3'UTR of the *cpxP* gene by RNase E in *Salmonella*, contributing to inner membrane protection in response to cell envelope stress (Chao & Vogel, 2016). Lately, SdhX, an sRNA processed from the 3'UTR of the *sdhCDAB-sucABCD* operon, which encode enzymes of the tricarboxylic acid (TCA) cycle, was found to negatively regulate the translation of acetate kinase (AckA) (De Mets et al, 2019) (Figure 14B). MicL is a  $\sigma^E$  dependent sRNA that regulates the expression of the major outer membrane lipoprotein Lpp in *E. coli*. Interestingly, it is transcribed from a promoter located within the coding region of the *cutC* gene (Figure 14C). The 308 nt primary transcript will then be processed to the 80 nt MicL sRNA (Guo et al, 2014b).

Finally asRNAs are RNAs transcribed from the complementary strand of another gene. Tens to thousands of asRNAs have been predicted by different RNA-Seq based approaches (Kawano et al, 2005; Thomason et al, 2015), leading to the concept of pervasive antisense transcription (Georg & Hess, 2018). It remains to determine how many putative asRNAs are physiologically

relevant, reflect a high level of background antisense transcription and/or inefficient transcription termination or are artefacts of the transcriptomic approach itself, be it at the level of library construction or bioinformatic analyses.

Thirdly, the existence of bifunctional sRNAs containing short ORFs make it difficult to distinguish sRNAs from mRNAs. This class of sRNAs can not only regulate mRNA expression but also encode proteins (peptides) to regulate different metabolite pathways (Gimpel & Brantl, 2017). RNA III is the first identified bifunctional sRNA (Balaban & Novick, 1995; Benito et al, 2000). As I mentioned above, RNAIII regulates several mRNA expression by base-pairing mechanism like most sRNAs. In addition, RNA III itself contains an ORF for cytotoxic peptide haemolysin  $\delta$ , which targets host cell membranes and causes cell lysis (Verdon et al, 2009).

SgrS is the first and unique bifunctional sRNA identified so far in Gram-negative bacteria. The expression level of SgrS RNA is highly induced under glucose-phosphate stress. As a base-pairing sRNA, it can negatively regulate the translation and stability of the PtsG mRNA, encoding the glucose transporter (Vanderpool & Gottesman, 2004). As a coding mRNA, it encodes a 43 amino acids (aa) peptide SgrT from an ORF located in the 5' portion of the sRNA. SgrT binds to the pre-existing PtsG protein and inhibits its activity (Wadler & Vanderpool, 2007). Three additional identified bifunctional sRNAs are: Psm-mec in *S. aureus* interacts with and inhibits AgrA mRNA translation and encodes a 22 aa hydrophobic,  $\alpha$ -helical peptide (Kaito et al, 2013; Kaito et al, 2011) (Table 2). Pel in *Streptococcus pyogenes* activates the transcription of different virulence factor encoding mRNAs and encodes a 53 aa haemolytic peptide (Mangold et al, 2004). SR1 in *B. subtilis* inhibits the translation of AhrC mRNA and encodes a 39 aa peptide called SR1P (Heidrich et al, 2006; Heidrich et al, 2007). In addition, several other sRNAs with known base-pairing functions and potential ORFs have been predicted (Gimpel & Brantl, 2017), indicating that the amount of bifunctional sRNA may be underestimated.

Table 2 Overview of experimental confirmed bifunctional sRNAs in bacteria

sRNAs	Length	Base pairing function(s)	Peptide encoded	Peptide function(s)	Bacteria (reference)
RNAIII	514 nt	Repressing the expression of early virulence genes and surface proteins and improving the production of late infectious toxins.	Hld (26 aa)	Cytotoxic peptide haemolysin $\delta$	<i>S. aureus</i> (Benito et al, 2000) (Verdon et al, 2009)
Psm-mec	157 nt	Inhibiting AgrA production and suppressing the fast, acute virulence of bacteria.	PSM-mec (22 aa)	Activating the biofilm formation	<i>S. aureus</i> (Kaito et al, 2013) (Kaito et al, 2011)
Pel	459 nt	Activating the transcription of virulence factor mRNAs like <i>emm</i> , <i>sic</i> and <i>nga</i> .	SLS (53 aa)	B-haemolysin streptolysin	<i>S. pyogenes</i> (Mangold et al, 2004)
SR1	205 nt	Inhibiting <i>ahrC</i> translation initiation. AhrC is the transcriptional activator of the arginine catabolic operon <i>rocABC</i>	SR1P (39 aa)	GapA/SR1P can affect the bacterial degradosome	<i>B. subtilis</i> (Gimpel et al, 2010) (Heidrich et al, 2006) (Heidrich et al, 2007)
SgrS	227 nt	Negatively regulating the translation and stability of PtsG mRNA.	SgrT (43 aa)	Repressing PtsG activity	<i>E. coli</i> (Wadler & Vanderpool, 2007)

The fast progress in the development of high-throughput cDNA sequencing technique, several RNA-Seq based approaches has allowed the development of the study of sRNAs in a lot of bacterias and pathogens. In *S. typhimurium*, 64 sRNAs were identified by high-throughput pyrosequencing (HTPS) technology (Sittka et al, 2008). In *Helicobacter pylori*, nearly 60 sRNAs were identified from the results of dRNA-Seq (Sharma et al, 2010). By combining the use of DETR'PROK and visual inspection of expression data, Liu *et al.* extended the number of *bona fide* sRNAs in *S. aureus* HG003 from 30 previously known (Bohn et al, 2010) to 50 (Liu et al, 2018b), whereas 303 putative trans-encoded sRNAs were identified in another RNA-seq study of *S. aureus* USA300 (Carroll et al, 2016). Another recent study identified 1583 putative regulatory RNAs in the Gram-positive soil bacterium *B. subtilis*, whose expression was assessed across 104 conditions (Bardill & Hammer, 2012; Perez-Reytor et al, 2016).

## 2 Marine pathogenic vibrios

*Vibrio* is a genus of Gram-negative bacteria, possessing a curved-rod shape and a polar flagellum. Species in this genus are ubiquitously distributed in the marine environment, ranging from the deep sea to shallow coastal water (Reen et al, 2006; Vesth et al, 2010). Vibrios can live in various ecological niches, either as free living forms, attached to biotic and abiotic surfaces or associated with hosts (Reen et al, 2006; Thompson et al, 2004).

Vibrios are responsible of severe infections both in human and animals. Some *Vibrio* species are human pathogens, notably particular serotypes of *V. cholerae* (causing cholera), *V. parahaemolyticus* (causing gastroenteritis) and *V. vulnificus* (causing wound infections and tissue necrosis) (Figure 15) (Thompson et al, 2004). In recent years, a worldwide increase of *vibrio*-associated human illnesses has been detected, probably because of the risen sea surface temperature, since the vibrios preferentially grow in warm (>15°C) seawater (Le Roux et al, 2016; Vezzulli et al, 2013). Besides, a wide range of diseases of marine animals, e.g., corals, bivalves, and fishes, have been linked to *Vibrio* infections (Figure 15) (Chakraborty et al, 1997). Particularly, vibrios have been suggested to be responsible in France of the “summer mortality syndrome” in oysters (*Crassostrea gigas*) that have resulted in losses of up to 80%-100% of the production certain years (Le Roux et al, 2015; Le Roux et al, 2016). Horizontal gene transfer (HGT) has contributed to several important characteristics of vibrios, including virulence traits acquisition (Hazen et al, 2010; Thompson et al, 2004). *V. cholerae* evolved from a free living environmental organism to a human pathogen by phage acquisition (Heidelberg et al, 2000). *V. crassostreae*, a member of the Splendidus clade (see

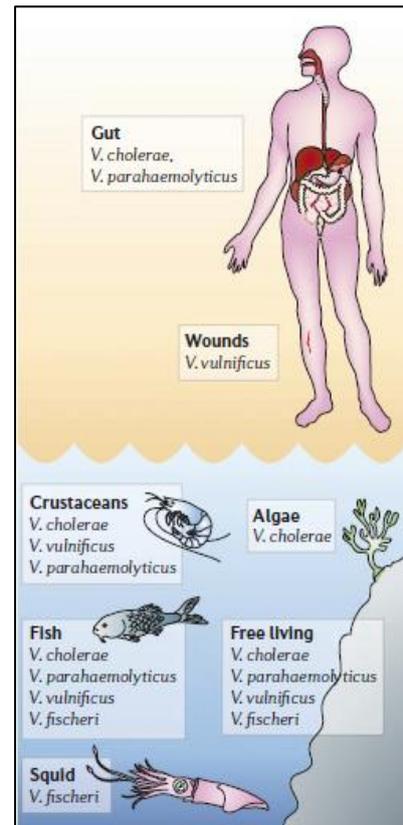


Figure 15 The wide range of niches of four *vibrio* species (Reen et al, 2006). All four *vibrio* species are aquatic and can colonize fish and marine invertebrates. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* infect humans. *V. fischeri* colonizes the squid, forming a symbiotic relationship.

Figure 15) (Chakraborty et al, 1997). Particularly, vibrios have been suggested to be responsible in France of the “summer mortality syndrome” in oysters (*Crassostrea gigas*) that have resulted in losses of up to 80%-100% of the production certain years (Le Roux et al, 2015; Le Roux et al, 2016). Horizontal gene transfer (HGT) has contributed to several important characteristics of vibrios, including virulence traits acquisition (Hazen et al, 2010; Thompson et al, 2004). *V. cholerae* evolved from a free living environmental organism to a human pathogen by phage acquisition (Heidelberg et al, 2000). *V. crassostreae*, a member of the Splendidus clade (see

below), comprises oyster virulent (*e.g.*, J2-9) and non-virulent (*e.g.*, J2-8) strains. Extra-chromosomal DNA analysis has shown that a mobilizable plasmid pGV1512, encoding virulence factors, is needed for the pathogenicity of the virulent strain J2-9 (Bruto et al, 2017).

*Vibrio* species all contain two chromosomes with different sizes. Chromosome I (larger size) carries most of the essential housekeeping genes (Heidelberg et al, 2000; Schoolnik & Yildiz, 2000) whereas the more variable chromosome II carries the majority of species-specific genes with unknown functions, and is thought to have evolved from a megaplasmid acquired before the diversification of the *Vibrionaceae* (Reen et al, 2006; Thompson et al, 2004). One of the advantages of having two separated chromosome is that they can be replicated simultaneously and independently, which can shorten the time of DNA replication (Yamaichi et al, 1999).

## 2.1 *Vibrio splendidus* clade

Phylogenetically, *Vibrio* species are divided into 22 distinct clades based on the analysis of 8 housekeeping genes (Sawabe et al, 2007; Sawabe et al, 2013a). Among them, the highly diverse *V. splendidus* clade consists of various *Vibrio* species (Figure 16A) that are ubiquitous in the marine coastal environment (Perez-Cataluna et al, 2016) and have been associated with epidemic outbreaks of aquatic animals (Gay et al, 2004a; Lopez-Joven et al, 2018; Munn et al, 2008; Nasfi et al, 2015). It has been shown that pathogenic vibrios of the *splendidus* clade, mainly *V. crassostreae* and *V. tasmaniensis*, as well as a herpes virus OsHV-1, are responsible for the recurrent mortalities of juvenile oysters (*C. gigas*) in Europe (Bruto et al, 2018; Gay et al, 2004a; Lemire et al, 2014; Segarra et al, 2010). Recent work has resolved the controversy regarding the culprit of mortalities, between a viral and a bacterial cause. It was shown that the disease starts by the OsHV-1 infection and multiplication, which alters the oyster immune system, further allowing bacterial colonization and septicemia leading to oyster death. (De Lorgeril et al, 2018a; De Lorgeril et al, 2018b).

*V. crassostreae* is particularly abundant in diseased oysters. The virulence of *V. crassostreae* is related to an ancestrally acquired gene *r5.7* (Bruto et al, 2018; Lemire et al, 2014) and a virulence plasmid (Bruto et al, 2017). R5.7, a 798 aa exported protein, is widely conserved

across the *splendidus* clade, and is essential but not sufficient for virulence (Lemire et al, 2014). The large mobilizable plasmid pGV1512, encoding virulence factors, is also needed to kill the oyster (Bruto et al, 2017). By using an integrative approach, Rubio *et al.* (Rubio et al, 2019) characterized the within-host cellular and molecular mechanisms leading to colonization success in oysters infected with *V. crassostreae*. It's shown that as an extracellular pathogen, *V. crassostreae* uses cell contact-dependent cytotoxicity to overcome the cellular defenses of its host and the *r5.7* gene is a key determinant of the bacterial cytotoxicity.

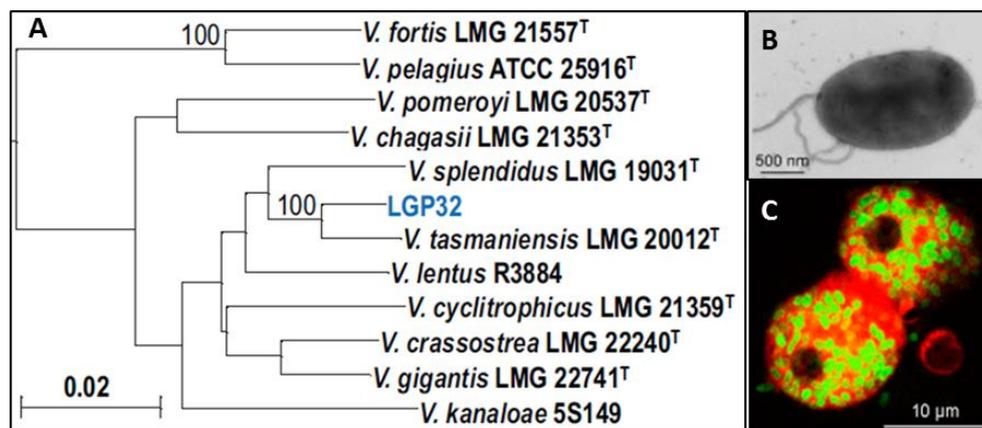


Figure 16 *Vibrio* species in the *V. splendidus* clade (A). The phylogenetic tree was built on the basis of 8 housekeeping genes (*gapA*, *gyrB*, *ftsZ*, *mreB*, *pyrH*, *recA*, *rpoA*, and *topA*) (Sawabe et al, 2013a). Transmission electron microscopy of LGP32 (B) (Vanhove et al, 2015). Evan's blue-stained oyster hemocytes (red) invaded by GFP-expressing LGP32 (green) (Duperthuy et al, 2011).

*V. tasmaniensis* LGP32 (Figure 16B) was isolated from the hemolymph of oysters suffering from outbreaks of summer mortality and was demonstrated to be pathogenic to oysters and clams when injected intramuscularly (Gay et al, 2004a). It is a facultative intracellular pathogen that invades the hemocytes, the oyster immune cells. It is resistant to oyster antimicrobial peptides (AMPs), oxidative stress and copper efflux (Figure 16C) (Duperthuy et al, 2011; Vanhove et al, 2016). Although a pathogen and a member of the Splendidus clade, it has lost the *r5.7* gene, and does not carry the virulence plasmid. However, several potential virulence factors have been identified in LGP32, including the secreted metalloprotease Vsm (Binesse et al, 2008; Le Roux et al, 2007), the invasion porine OmpU (Duperthuy et al, 2010) and an invasive

vesicular serine protease Vsp (Vanhove et al, 2015). Specifically, Vsm is the major toxicity factor in the extracellular products (ECP) of LGP32 (Binesse et al, 2008; Le Roux et al, 2007). The OMP OmpU contributes to the resistance of oyster antimicrobial peptides, attach to and invasion of oyster hemocytes and is essential for the virulence of LGP32 in oyster infection experiments (Duperthuy et al, 2010; Duperthuy et al, 2011). Vsp is a major gelatinase/caseinase of LGP32 present in outer membrane vesicles (OMVs) that deliver virulence factors to the host immune cells and protecting against antimicrobial peptides (Vanhove et al, 2015). Rubio *et al.* (Rubio et al, 2019) also characterized the within-host cellular and molecular mechanisms leading to colonization success in oysters infected with *V. tasmaniensis*. They found that *V. tasmaniensis* cytotoxicity is dependent on phagocytosis and requires intracellular secretion of type 6 secretion systems (T6SS) effectors. T6SS is a dynamic nanomachine present in many Gram-negative bacteria, it's used to inject toxic effectors into recipient cells (Nguyen et al, 2018b).

In 2012, the full transcriptome of LGP32 growing exponentially in rich medium was determined, leading to the identification of 471 putative regulatory 5' UTR RNAs, 250 *trans*-encoded sRNA candidates and a number of asRNAs (Toffano-Nioche et al, 2012), giving us an opportunity to study the roles of regulatory RNAs in this strain. **One candidate sRNA was chosen and its characterization is presented in Chapter II.**

## **2.2 *Vibrio alginolyticus***

*V. alginolyticus* (Figure 17) is an opportunistic pathogen of both human and marine animals. As a human pathogen, *V. alginolyticus* can cause otitis, endophthalmitis and wound infections after direct contact with contaminated seawater or consumption of raw seafood (Austin, 2010). It has been listed as one of the most common *vibrio* pathogens, together with *V. parahaemolyticus* and *V. vulnificus* in the United States (Jacobs Slifka et al, 2017; Newton et al, 2012).

As a marine animal pathogen, *V. alginolyticus* is responsible for hemorrhagic septicemia, skin discoloration, and necrotic lesions in the muscle tissue in various fish species, leading to mortality (Austin & Austin, 2016). The ECPs of *V. alginolyticus*, containing several hydrolytic proteases, have been shown to be lethal to grouper fish (*Epinephelus malabaricus*) (Lee, 1995)

and induced the production of hepatic heat shock proteins, playing critical roles in immune function and protection in sea bream (Deane et al, 2004). The toxicity of *V. alginolyticus* is mediated by the effectors Val1686 and Val1680 of a type III secretion system (T3SS), resulting in rapid apoptosis, cell rounding, and osmotic lysis of infected fish cells (Zhao et al, 2010; Zhao et al, 2018).

194 sRNA candidates had been predicted through RNA-Seq in the presence or absence of the RNA chaperon Hfq in *V. alginolyticus* ZJ-T, a translucent and smooth variant of ZJ-51 which was isolated from diseased *E. coioides* (Deng et al, 2016b). **One candidate sRNA was chosen for further study and its characterization is presented in Chapter I.**

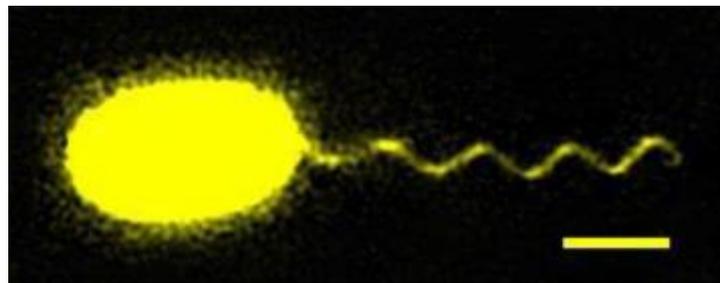


Figure 17 Representative high-resolution fluorescence image of *V. alginolyticus* taken by the structured illumination microscopy (Chen et al, 2017b). Scale bar, 1.5  $\mu$ m.

### 3 Regulatory small RNAs in the *vibrio* genus

Comparing with the sRNAs studied in enterobacteriaceae, *Vibrio* sRNAs are much less known. In addition, the majority of studies regarding sRNAs in vibrios have focused on *V. cholerae*, the cause of cholera pandemics, which has resulted in over 100,000 human deaths worldwide (Rosenberg, 1962).

The pathogenesis of *V. cholerae* has been studied for decades. Cholera results from the ingestion of contaminated food or water. Two primary virulence factors, cholera toxin (CT) and toxin co-regulated pilus (TCP), are responsible for the enterotoxicity and the colonization of the intestine, respectively (Childers & Klose, 2007). CT is encoded by the operon *ctxAB*, acquired from the lysogenic bacteriophage CTX $\phi$ , and leads to the loss of fluid from the host cell

(Waldor & Mekalanos, 1996). TCP is a type IV pilus encoded by the operon *tcpA-F*, which is located in the vibrio pathogenicity island (VPI) (Taylor et al, 1987). The expression of both *ctxAB* and *tcpA-F* are directly activated by the transcriptional factor ToxT, which is also encoded within the VPI (Figure 18) (Higgins et al, 1992). The transcription of *toxT* is activated by two transmembrane regulatory proteins: ToxRS and TcpPH (Childers & Klose, 2007; Krukonis et al, 2000). ToxRS is constitutively expressed whereas the transcription of *tcpPH* is regulated by environmental signals such as temperature and pH and two activator proteins, AphA and AphB (Carroll et al, 1997; Kovacicova et al, 2004; Thomas et al, 1995). Recently, ToxT was found to be directly regulated by an RNA thermometer located in the 5' UTR of its mRNA. This thermometer contains a four U anti-SD element that allows the ribosome to access to the SD sequence at 37°C but not 20°C (Figure 18) (Weber et al, 2014), facilitating *V. cholerae* virulence expression in the relevant environmental condition found in the human intestine.

### 3.1 sRNA discovery in vibrios

Initially, sRNAs were identified in *vibrio* based on sequence similarity with known sRNAs in *E. coli*. A systematic search for sRNAs by a bioinformatic study based on genomic DNA features such as the presence of Rho-independent terminator within intergenic regions, identified 32 novel sRNAs candidates. For 6 of them, their expressions were validated by northern blotting (Livny et al, 2005). This led to the identification of a RyhB homolog in *V. cholerae* (Davis et al, 2005). Similar to *E. coli* RyhB, VcRyhB is also Hfq dependent and its expression is repressed by Fur and highly induced under iron limiting condition (Davis et al, 2005; Masse & Gottesman, 2002; Mey et al, 2005). Although the central region of EcRyhB and VcRyhB are conserved, VcRyhB is significantly larger than EcRyhB (215nt VS 90nt) and the majority of the sRNA sequence is divergent (Davis et al, 2005). RyhB homologs were also found in *V. harveyi* and *V. parahaemolyticus* (Davis et al, 2005). Functionally, in addition to regulating iron utilizing genes such as *sodB*, *sdhC*, and *fumA* (encoding fumarate hydratase) as it does in *E. coli*, VcRyhB is also involved in the regulation of motility, chemotaxis and biofilm formation (Mey et al, 2005).

Since sRNAs are generally not conserved in primary sequences, even between closely related species, these screening approaches based on sequence homology are limited and were rapidly replaced by high-throughput sequencing-based approaches. In *V. cholerae*, over 500 putative intergenic sRNAs were identified (Liu et al, 2009). To identify sRNAs regulated by ToxT, the major transcriptional regulator of the virulence regulon, ToxT chromatin immunoprecipitation sequencing (ChIP-Seq) was carried out in a *V. cholerae* overexpressing ToxT (Bradley et al, 2011). As a result, 18 putative ToxT activated sRNAs were discovered. Besides, using differential RNA-seq, Mandlik *et al.* identified 77 putative novel sRNAs whose expression level changed during growth in the intestines of infant rabbits and infant mice compared to laboratory cultures (Mandlik et al, 2011).

Deep sequencing-based genome-wide sRNA identification have been carried out also in other vibrios, such as *V. alginolyticus* ZJ-T (Deng et al, 2016b), *V. tasmaniensis* LGP32 (Toffano-Nioche et al, 2012) and *V. parahaemolyticus* (Garcia et al, 2017), generating a reservoir of sRNA to be studied. Up to date, less than 20 of these sRNAs have been functional characterized (see table 3), demonstrating their involvement in the regulation of critical activities such as virulence, quorum sensing, and biofilm formation.

Table 3 Overview of experimentally characterized sRNAs in vibrios

Name	Regulated/induced by	Targets	Physiological functions	<i>Vibrio</i> species	References
<b><i>Trans-encoded sRNAs</i></b>					
<b>CsrBCD</b>	VarA	CsrA	Regulating quorum sensing, virulence via CsrA.	<i>V. cholerae</i> <i>V. tasmaniensis</i>	(Lenz et al, 2005; Nguyen et al, 2018a)
<b>MicX</b>	Abundant in the cell.	<i>vc0972</i> (-), <i>vc0620</i> (-)	Control of OMP synthesis and peptide transportation	<i>V. cholerae</i>	(Davis & Waldor, 2007)
<b>Qrr1-4</b>	Cell density	<i>luxO</i> (-), <i>hapR</i> (-), <i>vca0939</i> (+),	Quorum sensing control and de-repression of virulence genes.	<i>V. cholerae</i> <i>V. harveyi</i>	(Bardill et al, 2011; Lenz et al, 2004; Weber et al, 2011)

		<i>aphA</i> (+)		<i>V. anguillarum</i>	
<b>RyhB</b>	Fur	<i>sodB</i> , <i>sdhC</i> , <i>gltB1</i> , <i>fumA</i> , <i>acnA</i> , <i>ftn</i> , and <i>bfr</i> (-)	Iron homeostasis	<i>V. cholerae</i> <i>V. parahae-</i> <i>molyticus</i>	(Davis et al, 2005; Tanabe et al, 2013)
<b>Spot 42</b>	Constitutively expressed	VP1682 (T3SS chaperon protein) (-)	Cytotoxicity, sugar metabolism	<i>V. parahae-</i> <i>molyticus</i> <i>Aliivibrio</i> <i>salmonicida</i>	(Tanabe et al, 2015) (Hansen et al, 2012)
<b>Srvg23535</b>	Constitutively expressed	ABC transporter gene, chemotaxis protein encoding gene, <i>exsE</i>	Osmotic and pH stress resistant. Carbon and nitrogen sources utilization.	<i>V. alginolyticus</i>	(Deng et al, 2018)
<b>TarA</b>	ToxT	<i>ptsG</i> (-)	Regulating the glucose transportation.	<i>V. cholerae</i>	(Richard et al, 2010)
<b>TarB</b>	ToxT	<i>tcpF</i> (-)	Virulence	<i>V. cholerae</i>	(Bradley et al, 2011)
<b>TfoR</b>	Chitin induced	<i>tfoX</i> (+)	Regulating natural competence in response to chitin.	<i>V. cholerae</i>	(Yamamoto et al, 2011)
<b>VrrA</b>	$\sigma^E$	<i>vrp</i> , <i>ompA</i> (-), <i>ompT</i> (-)	Outer membrane porin synthesis.	<i>V. cholerae</i>	(Sabharwal et al, 2015; Song et al, 2008; Song et al, 2014; Song et al, 2010)
<b>VqmR</b>	VqmA	<i>vpsT</i>	Biofilm formation	<i>V. cholerae</i>	(Papenfort et al, 2015)
<b>Antisense sRNAs</b>					
<b>MtlS</b>	MtlA	<i>mtlA</i>	Mannitol Transportation	<i>V. cholerae</i>	(Chang et al, 2015; Mustachio et al, 2012; Zhang & Liu, 2019)
<b>RNA<math>\alpha</math></b>	Fur	<i>fatB</i> (-)	Regulating ferric siderophore anguibactin transportation	<i>V. anguillarum</i>	(Chen & Crosa, 1996)
<b>RNA<math>\beta</math></b>	Fur	<i>angRT</i> (-)	Control of Anguibactin biosynthesis	<i>V. anguillarum</i>	(Stork et al, 2007)
<b>Cis-regulatory RNA: thermometer</b>					
<b>5'UTR of ToxT</b>	20°C →37°C	<i>ctxAB</i> (+), <i>tcpA-F</i> (+), <i>tarA</i> (+), <i>tarB</i> (+)	Virulence	<i>V. cholerae</i>	(Weber et al, 2014)

“+” means positively regulated by the sRNA and “-” means negatively regulated by the sRNA.

### 3.2 sRNAs involved in the regulation of quorum sensing in vibrios

Bacterial group behaviors are controlled by a cell-to-cell communication system, called quorum sensing (QS). In response to changes in the cell density, QS enables bacteria to modulate the expression of dozens of genes that sustain various physiological activities (Abisado et al, 2018; Papenfort & Bassler, 2016; Whiteley et al, 2017). QS relies on the synthesis, release, and detection of small signaling molecules, called autoinducers (AIs) (Abisado et al, 2018; Herzog & Papenfort, 2018a). AIs are produced at a constant rate and thus, they accumulate in the environment as bacterial density increases. In Gram-negative bacteria, acyl-homoserine lactones (AHLs), synthesized by Lux-I type synthases, are the most common AIs (Liu et al, 2018a). In the 1960s and 1970s, the marine bacterium *V. fischeri* was found to auto-induce bioluminescence in high cell density cultures via QS system (Nealson & Hastings, 1979). At high cell density, the accumulated AHLs interact with and activate LuxR transcriptional factor. The active LuxR, in turn, promotes the expression of the *luxCDABEG* operon, which contains genes responsible for bioluminescence (Abisado et al, 2018).

Similar QS systems have been characterized in several vibrios, highlighting the role of several sRNAs in modulating this complex regulatory network (Figure 18).

In *V. cholerae*, three AIs have been discovered up to date (Figure 18). Cholera autoinducer 1 (CAI-1, (S)-3-hydroxytridecan-4-one), produced by CqsA, is recognized by the membrane bound protein CqsS (Higgins et al, 2007). AI-2 ((2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate), synthesized by LuxS synthase, is sensed by the membrane bound protein complex LuxPQ (Chen et al, 2002). Recently, a third AI in *V. cholerae* was found: DPO (3,5-dimethylpyrazin-2-ol), which is made from threonine and alanine with the aid of threonine dehydrogenase (Tdh) (Papenfort et al, 2017). Different from CqsS and LuxPQ, the DPO receptor protein VqmA is a cytoplasmic protein.

At low cell density (LCD) and low AIs concentration, the receptors CqsS and LuxQ act as kinases, channeling phosphate to the phosphotransfer protein LuxU, which passes it to the response regulator LuxO (Bassler et al, 1994). Phosphorylated LuxO, together with the sigma

factor  $\sigma^{54}$  induces the transcription of four homologous sRNAs Qrr1-4 (2-5 copies in other vibrios) (Lilley & Bassler, 2000). Each Qrr contains a 21 nucleotide region conserved among vibrios, and regulates a set of mRNA targets aided by the RNA chaperone Hfq, either positively or negatively (Bardill et al, 2011; Feng et al, 2015). Specifically, the Qrr sRNAs positively regulate the expressions of AphA, an activator of genes required for biofilm formation and for pathogenicity (Rutherford et al, 2011) and *vca0939*, encoding the diguanylate cyclase that synthesizes the intracellular second messenger cyclic-di-GMP (Hammer & Bassler, 2007; Zhao et al, 2013), by relieving an inhibitory structure in the RBS region. On the other hand, Qrr sRNAs negatively regulate expression of the transcriptional regulators HapR (Bardill et al, 2011) and LuxO (Svenningsen et al, 2009) by base-pairing with the 5' UTR and occluding the RBS. HapR is an important regulator that regulates a variety of activities in *V. cholerae*, including virulence by repressing the expression of AphA (Lin et al, 2007) and biofilm formation by inhibiting the production of another transcriptional factor VpsT, which drives the expression of polysaccharide synthesis genes and other protein components for biofilm assembly (Waters et al, 2008). Also, VpsT requires the second messenger c-di-GMP for full activity, which is synthesized by Qrr activated enzyme (Krasteva et al, 2010). Overall, at LCD, Qrr sRNAs are induced, AphA is produced while HapR is not.

At high cell density (HCD), binding of AIs to the receptors CqsS and LuxQ convert them from kinases to phosphatases, leading to dephosphorylation and inactivation of LuxO and resulting in cessation of Qrr expression (Herzog & Papenfort, 2018b). Reduced production of the Qrrs results in increased HapR production and inhibition of AphA synthesis. With HapR being produced, virulence gene expression and biofilm formation of *V. cholerae* are inhibited. On the other hand, accumulated DPO binds to the specific receptor protein VqmA and the active DPO-VqmA complex induces the expression of the sRNA VqmR. In concert with Hfq, VqmR base-pairs to and represses the expression of *vpsT* mRNA, further inhibiting biofilm formation in *V. cholerae* (Papenfort et al, 2017).

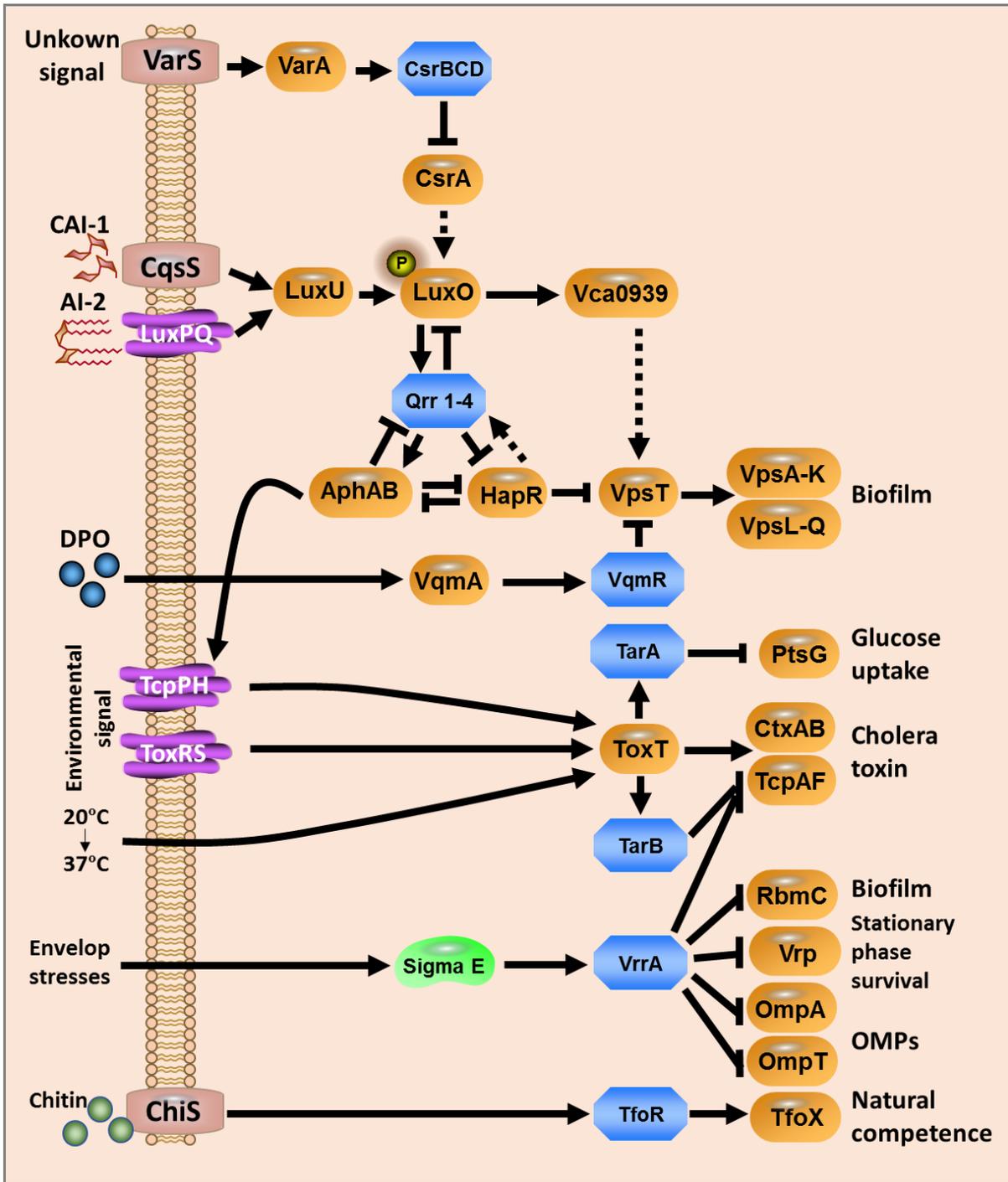


Figure 18 sRNAs regulatory network in *V. cholerae*. The mechanism is explained in the text.

The Qrr sRNAs negatively regulate expression of *luxO* mRNA, establishing a feedback loop to control Qrr production (Svenningsen et al, 2009). Several other regulatory feedback loops were characterized in the *Vibrio* Qrr regulatory network. For example, AphA represses the transcription of both the *qrr* sRNAs and *hapR* (Rutherford et al, 2011), HapR itself activates Qrr

transcription by an indirect mechanism that requires LuxO~P but does not involve direct interaction of HapR protein with the Qrr promoters (Svenningsen et al, 2008). These feedback loops contribute to fine-tuning the QS systems.

*V. cholerae* QS system is also regulated by an additional set of sRNAs CsrBCD, which are functionally homologous to the CsrBC sRNAs from *E. coli* (Lenz et al, 2005). The TCS controlling *V. cholerae* *csrBCD* expression, VarS/A, is homolog of BarA/UvrY in *E. coli*. Activated CsrBCD sRNAs bind and sequester CsrA which itself indirectly positively regulates the production of Qrr sRNAs through LuxO and affect the virulence and biofilm formation in *V. cholerae* (Lenz et al, 2005).

### 3.3 ToxT regulated sRNAs: TarA and TarB

Two sRNAs, TarA and TarB (ToxT activated RNAs) were found to be regulated by ToxT, the master virulence regulator (Bradley et al, 2011; Richard et al, 2010). Both of them are encoded within the VPI and activated by ToxT (Figure 18).

TarA ( $\approx$ 91nt) is a highly conserved sRNA among *V. cholerae* strains, containing two tox-boxes for ToxT binding within its promoter (Richard et al, 2010). Both microarray analysis and quantitative PCR results have shown that the *ptsG* mRNA level increased significantly in  $\Delta tarA$  and a base-pairing region was predicted in the 5' region of the *PtsG* mRNA. *ptsG* encodes the major glucose transporter and is negatively regulated by the sRNA SgrS in *E. coli* (Vanderpool & Gottesman, 2004). However, SgrS (227nt) is much larger than TarA and has no homolog in vibrios. Phenotypically, cells overexpressing TarA grow poorly in glucose-minimal media and  $\Delta tarA$  is compromised for infant mouse colonization when in competition with the wide type strain (Richard et al, 2010). This study sheds light on the relation between metabolic conversion and virulence in the pathogen.

Using sRNA-Seq associated with genome wide binding site study of ToxT, TarB was identified together with other 17 potential sRNAs (Bradley et al, 2011). Similar to TarA, two ToxT binding sites were found within the promoter region and the expression of TarB increased dramatically upon overexpression of ToxT. TarB minus strain outcompeted the WT strain when

colonizing the infant mouse intestine, suggesting that TarB is a negative regulator of virulence. Indeed, TarB negatively regulated the secreted colonization factor TcpF by base pairing to the 5' UTR region of the mRNA, in an Hfq independent manner. It was already known that the virulence factor ToxT can directly activate the expression of TcpF (Higgins et al, 1992). It is unclear why the ToxT positively regulated sRNA TarB would repress TcpF. Probably, TarB is also induced under unknown conditions, to fine-tune the virulence of *V. cholerae*.

### **3.4 Outer membrane protein synthesis regulatory sRNAs**

One of the strategies used by bacteria to survive in different environments is to modify their OMPs. As I mentioned above, in *E. coli*, a number of sRNAs are involved in the regulation of OMPs synthesis, such as OmrA, OmrB (Guillier & Gottesman, 2008), RybB (Balbontín et al, 2010) and MicA (Coornaert et al, 2010). In *V. cholerae*, two sRNAs were found to target sRNAs encoding OMPs.

The first one is MicX, identified using a bioinformatic algorithm that detects potential sRNAs within intergenic sequences (Livny et al, 2005). MicX appears to be a direct negative regulator of *vc0972*, which encodes an uncharacterized OMP, and *vc0620*, which encodes the periplasmic component of a peptide ABC transporter (Davis & Waldor, 2007) and Hfq is apparently not required for MicX's interactions with these targets.

The second OMP regulation sRNA in *V. cholerae* is the 140 nt sRNA VrrA, whose expression requires the membrane stress sigma factor  $\sigma^E$ , suggesting that VrrA plays a role in response to stress (Song et al, 2008). It has been shown that VrrA directly represses the translation of OmpA and OmpT by base-pairing with the SD sequence and inhibiting the access of ribosomes to these mRNAs (Song et al, 2008; Song et al, 2010). The repression of OmpA mRNA translation by VrrA is Hfq independent whereas Hfq is required for the repression of OmpT. Changes in the outer membrane protein composition in Gram-negative bacteria can affect the formation and release of OMVs (Sonntag et al, 1978). Consistent with this result, the decreased level of OmpA led to increased production of OMVs, thereby modulating the colonization ability of *V. cholerae* (Song et al, 2008).

In addition to OmpA and OmpT, the main colonization factor TcpA (Song et al, 2008), biofilm matrix protein RbmC (Song et al, 2014) and ribosome binding protein Vrp (Sabharwal et al, 2015) were found to be negatively regulated by VrrA through the same mechanism. VrrA is partly complementary to the 5' UTR of these mRNAs, and may inhibit the translation by base pairing and blocking the loading of the ribosome on the RBS. Vrp is a homolog of the ribosome associated inhibitor A (RaiA) of *E. coli*, is highly expressed during stationary phase and associates with the ribosome of *V. cholerae* (Sabharwal et al, 2015). Therefore, VrrA is an important sRNA involved in the regulation of OMP synthesis, virulence, biofilm formation and stationary survival in *V. cholerae* (Figure 18).

### **3.5 Other *vibrio* sRNAs**

Other characterized sRNAs in vibrios are listed in table 3. Up to date, the total number of sRNAs characterized in vibrios is still very small (less than 20). Deep sequencing and bioinformatics analysis had provided us a list of potential sRNAs in several vibrios pathogenic to species of aquaculture interest (Toffano-Nioche et al, 2012); (Garcia et al, 2017); (Deng et al, 2016b). But none of them have been yet functionally characterized. It is still a long way to go to understand the function of these sRNAs in *Vibrio* physiology.



# OUTLINE OF THE THESIS

The *Vibrionaceae* constitutes a family of marine bacteria of which many are responsible for infections affecting not only humans but also fish and marine invertebrates, representing the major cause of mortality in farmed marine species. They are able to colonize many habitats, existing as planktonic forms, biofilms or associated with various hosts. This high adaptability is linked to their capacity to generate genetic diversity, in part through lateral gene transfer, but also by diverse gene expression regulation. Bacterial regulatory small RNAs, usually 50-300 nt long, act by base-pairing with specific mRNA targets, affecting their translation and/or stability, and are important elements which regulate a variety of processes, including carbohydrate and intermediate metabolism. In this thesis, I will present you two distinct RNA regulatory mechanisms in two different *Vibrio* species.

- 1) In collaboration with Yiqin Deng, I explored the regulation of the biosynthesis of branched-chain amino acids (BCAAs: L-Leucine, L-Valine and L-Isoleucine) in *V. alginolyticus*, a marine fish and shellfish pathogen and an emerging opportunistic human pathogen. In this species, the *ilvGMEDA* operon encodes the main pathway for biosynthesis of BCAAs. Its upstream regulatory region shows no sequence similarity to the corresponding region in *E. coli* or other *Enterobacteriaceae* and yet, we show that this operon is regulated by a translated leader peptide (**Chapter I**).
- 2) *V. tasmaniensis* is a facultative intracellular pathogen, which can survive and multiply within hemocytes, the oyster immune cells. A previous transcriptomic study of *V. tasmaniensis* LGP32 led to the identification of hundreds of sRNAs in this species. Among them, Vsr217 was found to be conserved within vibrios, upstream of the *malk* gene which encodes a subunit of the major maltose transporter, and highly upregulated during oyster infection. Vsr217 is further studied and its characterization is presented in **Chapter II**.

# **RESULTS**

# CHAPTER I

## **The *ilvGMEDA* operon is regulated by transcription attenuation in *Vibrio alginolyticus* ZJ-T**

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## Abstract

Bacteria synthesize amino-acids according to their availability in the environment or within the host in the case of pathogens. We explored the regulation of the biosynthesis of branched-chain amino acids (BCAAs: L-Leucine, L-Valine and L-Isoleucine) in *Vibrio alginolyticus*, a marine fish and shellfish pathogen and an emerging opportunistic human pathogen. In this species, the *ilvGMEDA* operon encodes the main pathway for biosynthesis of BCAAs. Its upstream regulatory region shows no sequence similarity to the corresponding region in *Escherichia coli* or other Enterobacteriaceae and yet, we show that this operon is regulated by transcription attenuation. The translation of a BCAA rich peptide encoded upstream of the structural genes provides an adaptive response similar to the *E. coli* canonical model. This study of a non-model Gram-negative organism highlights the mechanistic conservation of transcription attenuation despite the absence of primary sequence conservation.

## Keywords

*Vibrio alginolyticus*, branched-chain amino acids (BCAAs), transcription attenuation, *ilvGMEDA* operon, leader attenuator, acetoxy acid synthase (AHAS)

## Introduction

Bacterial gene regulation has been extensively studied in model organisms such as *Escherichia coli* or *Bacillus subtilis*, uncovering many layers of exquisitely complex regulatory pathways at all levels of genome expression: transcriptional, translational, protein stability and activity levels, involving regulatory proteins and non-coding sRNAs as well as end-products. In the case of carbon compounds utilization, cells need to turn on the catabolic pathway only when the compound is present in their environment. On the contrary, in the case of essential compounds such as amino-acids (AAs), the genes encoding their biosynthetic pathways are expressed only when necessary, i.e. when these AAs cannot be scavenged from the environment. The biosynthetic pathway for branched-chain amino acids (L-isoleucine, L-leucine, L-valine: ILV or BCAAs) has been worked out in the 1950s by H. Edwin Umbarger in *E. coli*, who went on to explore its regulation (reviewed in (Salmon et al, 2006a; Willins & Calvo, 1992). This biosynthetic pathway is presented in Fig. 1A. In *E. coli* and other Enterobacteriaceae, the 15 *ilv* structural genes are organized in five transcription units: *leuABCD*, *ilvGMEDA*, *ilvBN*, *ilvIH* and *ilvC*. An additional gene *ilvY*, upstream of *IlvC* and transcribed in the opposite direction, encodes a transcriptional regulator of *ilvC* (Wek & Hatfield, 1986).

A first level of regulation is feed-back inhibition of the first pathway-specific enzyme by the end product of the pathway: L-leucine inhibits isopropylmalate synthase, encoded by *leuA*, L-valine inhibits the activity of acetolactate synthases encoded by *ilvIH* and *ilvBN* and L-isoleucine inhibits the activity of threonine deaminase encoded by *ilvA*. A second regulatory level is gene expression. At the transcriptional level, the global dual regulator Leucine Responsive Protein (Lrp) was found to regulate the *ilvBN* transcription unit (for a review see (Newman & Lin, 1995), whereas the *leuABCD* and the *ilvGMEDA* operons are essentially regulated by transcriptional attenuation (Hauser & Hatfield, 1983; Hauser & Hatfield, 1984; Lawther et al, 1987; Nargang et al, 1980; Searles et al, 1983; Wessler & Calvo, 1981): a short leader peptide that contains a high number of the regulating amino acid (Ile, Val, Leu) is encoded upstream of the operon between the promoter and the first structural gene. Each leader region is followed by a

transcription terminator site, the attenuator. The short polypeptide sequence monitors the cellular supply of amino-acylated tRNAs for the regulatory codons. When the AAs are in ample supply, the leader peptide is synthesized without interruption. In these conditions, the terminator forms and transcription termination is maximum, preventing expression of the downstream genes in the operons. If the AAs are in short supply, translation of the leader peptide is impeded. In such case, the untranslated leader transcript can form an alternative secondary structure that preempts the formation of the transcriptional terminator, allowing transcription to proceed through the operon (reviewed in (Salmon et al, 2006a)).

More recently, attention has turned to regulation of BCAA biosynthesis in Gram-positive bacteria, such as *B. subtilis*, *Lactobacillus* sp or *Staphylococcus aureus* (for a review see (Brinsmade, 2017)). In these species, *ilv* and *leu* genes are organized in three genetic loci, *ilvBH* and *leuABCD* being part of a single transcription unit. Both transcription units are transcriptionally regulated by the CodY transcriptional repressor which becomes active upon binding of either BCAA, at least *in vitro* (Guedon et al, 2001). In the case of *S. aureus*, *in vivo* CodY seems to respond specifically to the presence of Ile (Kaiser et al, 2018). In addition, regulation at the level of transcription attenuation by an attenuator peptide rich in Leu codons has also been demonstrated (Kaiser et al, 2018). Hence, in *S. aureus*, there are two levels of regulation, each one responding specifically to the presence of two different BCAAs. CodY is also an important regulator of virulence (Brinsmade, 2017), providing a link between BCAA biosynthesis and pathogenicity, reflecting the importance of dealing with possible scarcity of these AAs in the host.

In Gram-negative bacteria, studies have concentrated on the model organisms *E. coli* and *Salmonella typhimurium*. However, a comparative genomic analysis in proteobacteria suggested that regulation by transcription attenuation of BCAA synthesis is widely conserved in this phylum, including in three *Vibrio* species (Vitreschak et al, 2004). In addition, it has been shown that Lrp from *Vibrio cholerae* could compensate partially for the absence of Lrp in *E. coli* (Lintner et al, 2008).



## Results and discussion

### Two acetolactate synthases are involved in BCAAs biosynthesis in *V. alginolyticus*

Acetolactate synthase (AHAS) is the first common enzyme in the pathways leading to L-isoleucine, L-leucine and L-valine (Wek et al, 1985) (Fig. 1A). It is made of two polypeptides, a catalytic and an activating subunit. In *E. coli*, three pairs of genes, *ilvBN*, *ilvGM* and *ilvIH* encode AHAS I, AHAS II and AHAS III, respectively (reviewed in (Salmon et al, 2006a)). *ilvB*, *ilvGM* and *ilvIH* were detected on the chromosome I of *V. alginolyticus* ZJ-T, while the downstream gene of *ilvB* has no homology to *ilvN* but, instead, has high similarity to *fabG2*, which encodes a 3-oxoacyl-ACP-reductase (Fig. 1A-B). To determine which acetolactate synthases were responsible for BCAAs synthesis in *V. alginolyticus* ZJ-T, we constructed three deletion mutants:  $\Delta ilvGM$ ,  $\Delta ilvIH$  and  $\Delta ilvB$  as well as their various combination and examined the auxotrophy for ILV in these mutants (Fig. 2). In minimal medium supplemented by ILV, all mutants grew as well as the wild-type strain, whereas in absence of ILV, no growth was observed in the double mutant *ilvGM ilvIH*. Further, deleting only *ilvGM* had a significant effect on growth in absence of ILV whereas deleting *ilvIH* had no effect on auxotrophy. Deleting *ilvB* had no effect, either alone or in combination with other mutations. We concluded that *IlvGM* is the main acetolactate synthase in *V. alginolyticus*, and that, in its absence, *IlvIH* takes over, although not as efficiently. Finally, *ilvB* does not contribute to the cell acetolactate synthase activity.

### Existence of an *IlvL* leader peptide encoded upstream of the *IlvGMEDA* operon in *V. alginolyticus*.

In *E. coli*, the *ilvBN* operon is mostly regulated at the transcriptional level by the Leucine responsive protein (Lrp) in response to the presence of leucine and repressed by the transcriptional silencer H-NS. In addition, it is subjected to the stringent response mediated by ppGpp in response to amino-acid starvation (Baccigalupi et al, 1995; Levinthal et al, 1994; Willins & Calvo, 1992). On the contrary, the *ilvGMEDA*, *ilvBN* and *leuABCD* operons are regulated in

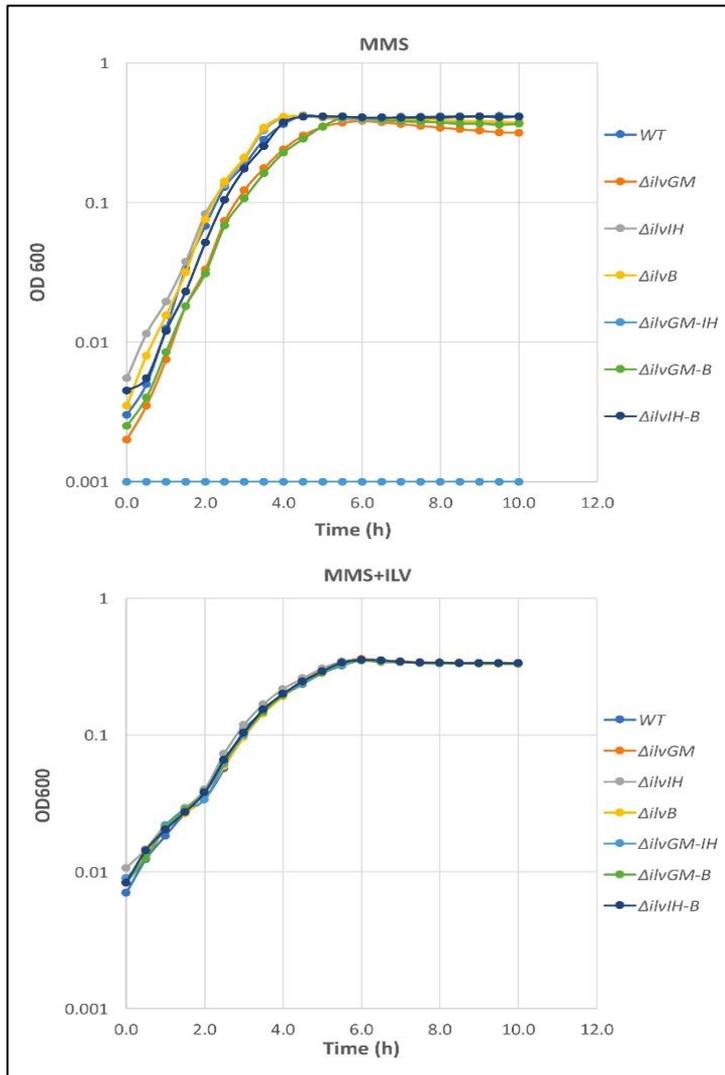


Fig. 2. Growth of wild type *V. alginolyticus* ZJ-T and *ilvGM*, *ilvIH* and *ilvB* derivative mutants in various combination. Cells from strains as indicated on the right were grown in MMS (upper panel) and MMS supplemented by BCAAs (lower panel).

response to the presence or absence of BCAAs by transcriptional attenuation (Friden et al, 1982; Lawther et al, 1987; Wessler & Calvo, 1981). Transcriptional attenuation is mediated by coupling the translation of a small peptide rich in BCAAs with the formation of an attenuator, leading to transcription termination upstream of the structural genes (reviewed in (Salmon et al, 2006a). The BCAA rich leader peptide is encoded between the operon promoter and the first structural gene of the operon.

We investigated if, in *V. alginolyticus*, the *ilvGMEDA* operon could also be regulated by a transcriptional attenuation

mechanism. Alignments of the *ilvG* gene upstream region with the corresponding regions in *E. coli* and other Enterobacteriaceae showed no conservation at the primary sequence level. However, we identified a small open reading frame encoding a putative 29 AA peptide with 13 BCAAs (Fig. 3A), although again no conservation of the AA sequence with *E. coli* IlvL could be detected, other than the enrichment in BCAAs. The 5' and 3' ends of the putative transcript were determined by 5' and 3' RACE. In minimal medium with salt (MMS), a single 5' end was identified 59 nt upstream of the putative leader peptide start codon (Fig. 3A) as well as a unique

3' end upstream of a putative transcriptional attenuation structure (Fig. 3B). We concluded this transcript had the potential to encode a BCAA rich leader peptide that we called IlvL.

To further localize the promoter of the *ilvLGMEDA* operon, and confirm the functionality of the terminator, we inserted in the multicopy plasmid pSCT32-*gfp* (Table 1) various DNA fragments from the upstream region of *ilvG* fused to a *gfp* reporter gene devoid of its own promoter but retaining 24 nt upstream of the ATG initiation codon, including the RBS, to generate transcriptional fusions. Plasmids carrying these fusions were introduced in *E. coli*, and relative fluorescence units were determined along growth (*cf.* Materials and Methods). As can be seen in Fig 3C, Gfp activity was maximal at the onset of growth. The region encompassing 53 nt upstream of the putative transcription start site (TSS) identified by 5' RACE had a strong promoter activity (Fig. 3C), confirming the presence of the  $\sigma^{70}$  promoter predicted by BPROM (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>) in this region. This activity further increased when 94 more upstream nt were included. Interestingly, this more upstream region was predicted to contain a Lrp binding site (Fig 3A). Lrp could contribute to the regulation of the operon at the transcriptional level, since its activity can be modulated by the binding of Leucine, and it is known to regulate the *ilvBN* operon in *E. coli* (Newman & Lin, 1995).

Including the putative terminator [-147, +303] in the fusion led to a strong decrease of the transcription of the downstream *gfp* gene, confirming the presence of a transcriptional terminating activity upstream of *gfp*. Deleting the predicted attenuator (*ilvL $\Delta$ att*) led to a 2-fold increase of transcription (Fig. 3C). However, the construct with  $\Delta att$  did not reach the *gfp* expression level observed in the fusion carrying only the promoter region, suggesting that more features in the *ilvL* coding region contribute to transcription attenuation than just this terminator. Finally, we deleted the *ilvL* initiation codon (*ilvL $\Delta$ ATG*) to investigate the requirement for *ilvL* translation and found a total absence of downstream transcription in the *ilvL $\Delta$ ATG* mutant.

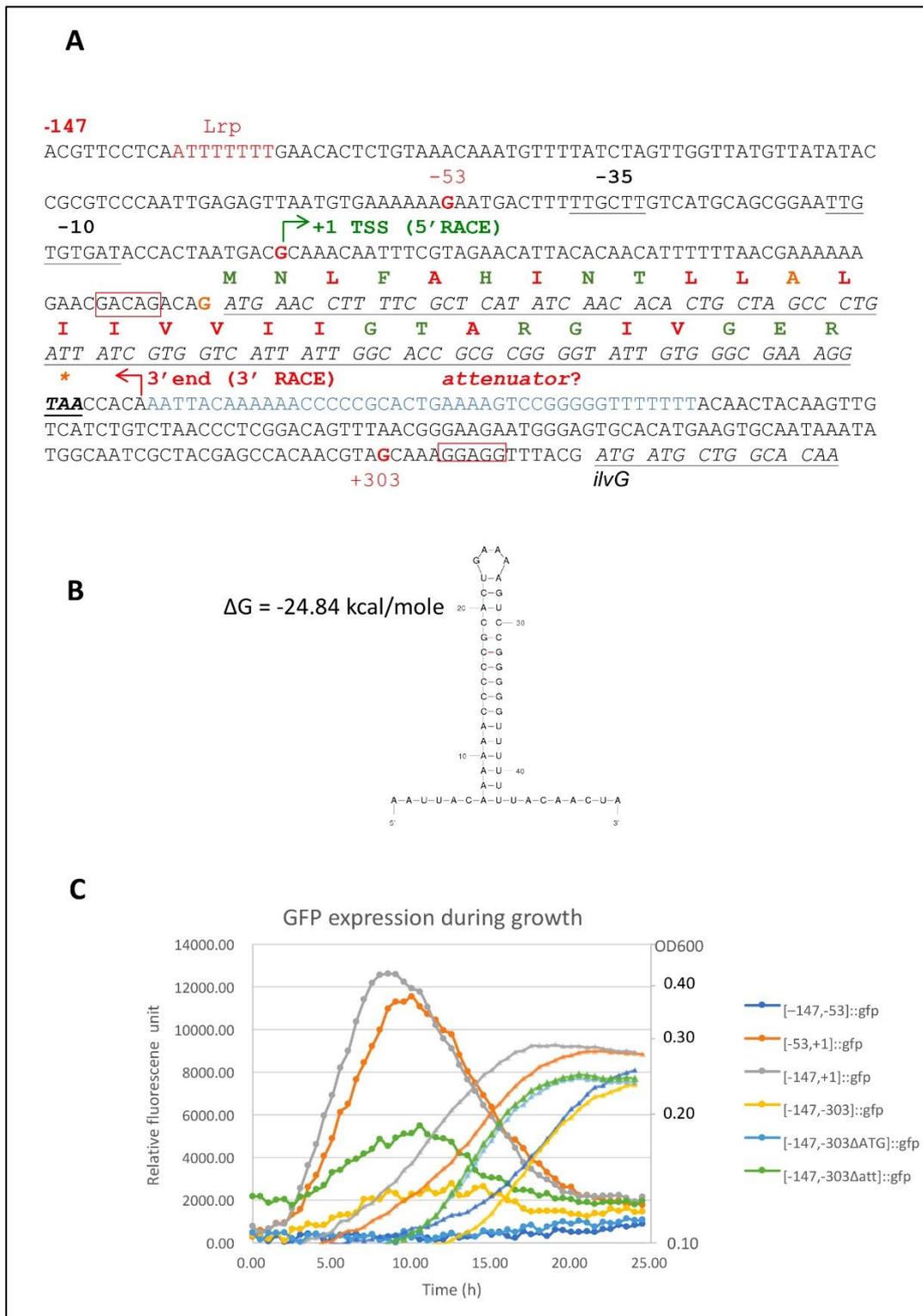


Fig. 3. Structure of the *ilvG* upstream region and transcription termination in *V. alginolyticus*. A- Sequence of the upstream region of the *ilvG* gene. A putative +1 transcription start site (TSS) as identified by 5'RACE is indicated and is numbered as +1. The 3' end of the leader transcript as determined by 3' RACE is also indicated. The predicted -10 and -35 of a sigma70 promoter are underlined and an upstream Lrp binding site is indicated in red. The open reading frame for the IlvL leader peptide is underlined, and the peptide sequence is indicated

above the nucleotide sequence. BCAAs are colored in red. The sequence corresponding to the potential transcription terminator/attenuator is in blue. The RBS for *ilvL* and *ilvG* are boxed in red. B- Secondary structure of the *ilvL* attenuator as predicted by RNA-fold ([ma.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi](http://ma.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi)). C- Various length DNA fragments from the upstream region of *ilvG* (coordinates corresponding to Fig. 3A with +1 = *ilvL* RNA 5' end) were fused to a promoter less *gfp* gene retaining its RBS. The resulting plasmids were introduced in *E. coli* BL21 and fluorescence was measured every 15 min during growth in M63. Results shown (primary axis : relative fluorescence units, filled circles, secondary axis: OD600, filled triangles) are the mean of three biological replicates.

### ***IlvGMEDA* transcription attenuation is induced by BCAAs**

Results obtained using transcriptional fusions are consistent with a model where the structural *ilv* genes transcription is regulated by transcription termination, which is itself coupled to *ilvL* translation. To confirm this model, we carried out northern blot experiments in *V. alginolyticus* itself. Specifically, we tested the effect of adding all three BCAAs or any one of them on transcription of the *ilv* genes, 30 mn after the addition to prevent variation of internal concentration due to the cells synthesizing these AAs. We also included *ilvI*, coding for the AHASIII large subunit in our analysis, because we wanted to know if, despite playing a minor role in BCAAs synthesis, its expression was also repressed by the presence of BCAAs.

Probing *ilvL* showed that the *ilvL* transcript level was not affected by the BCAAs, confirming that the regulation by these AAs is not at the level of the *ilvGMEDA* promoter. In contrast, *ilvG*, *ilvA* and *ilvI* transcription was inhibited by the addition of all three BCAAs. In the case of the *IlvGMEDA* operon, valine alone seems to be the most efficient, whereas in the case of the *ilvIH* operon, leucine was the strongest inhibitor (Fig. 4B and 4C).

In absence of externally added BCAAs, *V. alginolyticus* is producing these AAs to restore their internal concentration. This will contribute to downregulate expression of the biosynthetic genes even in absence of ILV in the medium. Upon inactivation of the *ilvGM* genes, encoding the main acetolactate synthase activity, the cells are still able to grow in non-supplemented minimal medium, thanks to the residual activity of *IlvIH*, but a reduction of internal BCAA concentration could be expected. In keeping with this hypothesis, a strong accumulation of the *ilvEDA* mRNA was observed in the  $\Delta ilvGM$  mutant (Fig. 4D). As expected, deleting *ilvGM* did

not affect the overall functioning of the regulatory system since adding ILV to the medium still repressed the expression of *ilvEDA* in the mutant.

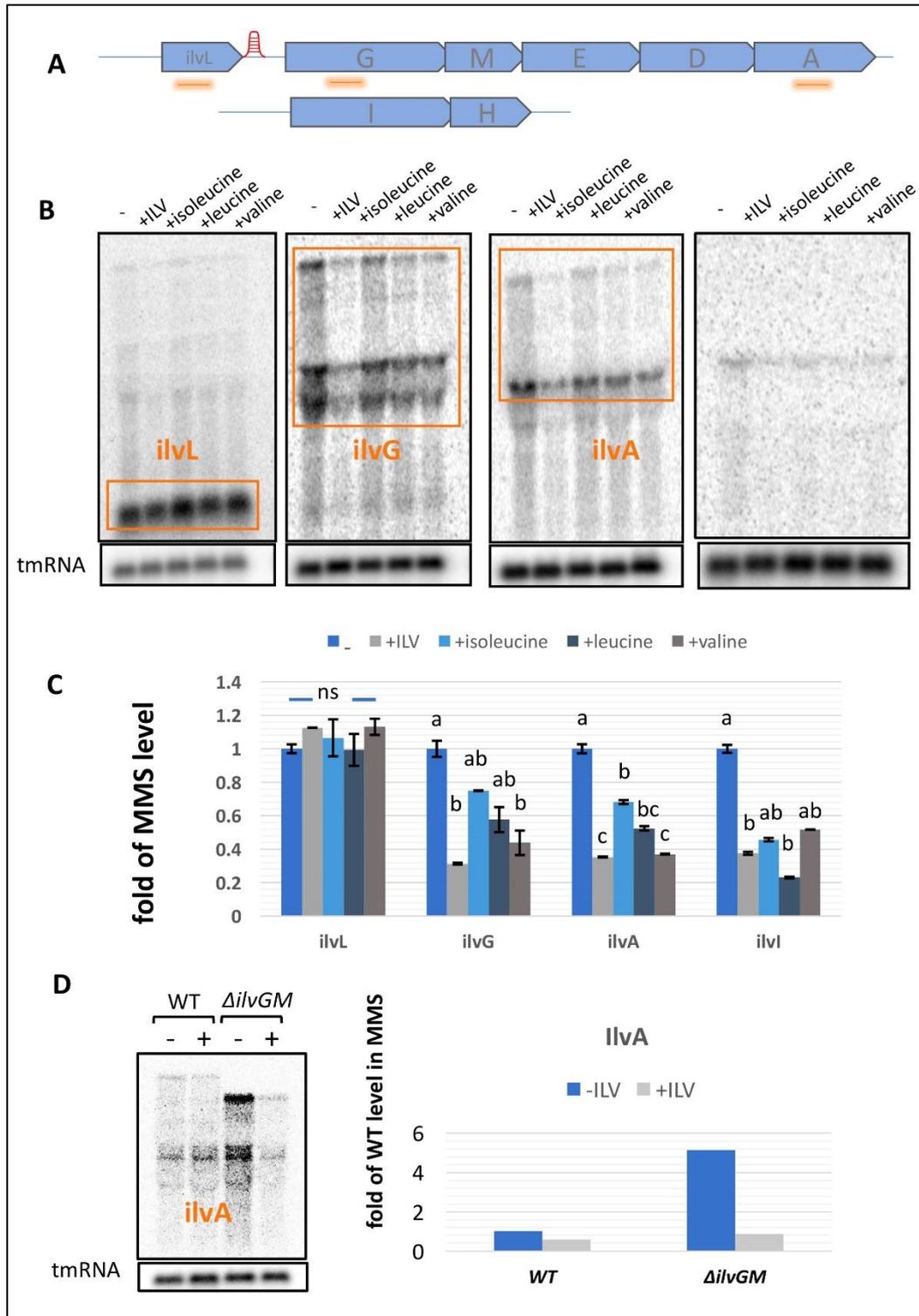


Fig. 4. Effect of BCAAs on expression of *ilv* genes. *V. alginolyticus* ZJ-T was grown in MMS until  $OD_{600} \approx 0.3$  before incubation for 30 min in the same medium supplemented or not by BCAAs as indicated. Gene expression was then measured by Northern blotting,

as described in Materials and Methods. A- Approximate position of the probes used. B- Northern blots. The rectangles indicate the zones in the blot that were used for quantification. tmRNA was used to monitor loading. C-Quantification of the results in Fig. 4B. The signal was first normalized to tmRNA. Then, for each probe, expression levels are expressed relative to the level observed in the absence of BCAAs. Values represent averages of data from two independent experiments, with error bars corresponding to SEM. Data were analyzed by one-way ANOVA followed by pairwise *t* tests. Values considered to be significantly different in the test ( $P<0.05$ ) are indicated by different letters. D- Effect of deleting the *ilvGM* genes on expression of the *ilvGMEDA* operon. Left: northern blots as in 4B, probing for *ilvA*. Right: quantification was as in 4C.

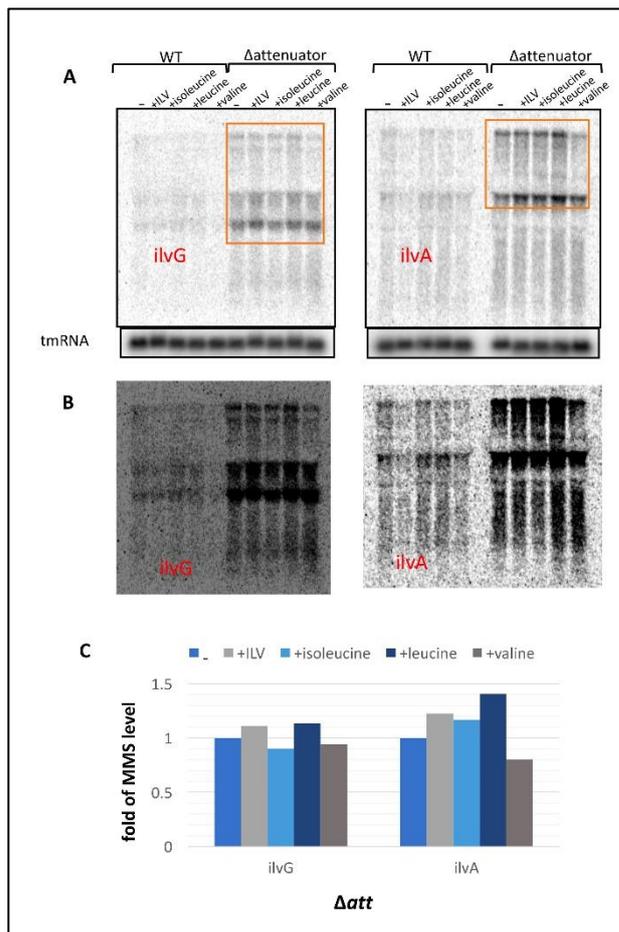


Fig. 5. Effect of attenuator deletion on expression of the *ilvG* and *ilvA* genes. A- Northern blots and their quantification were as in Fig. 4. B- Overexposure of the blots presented in Fig. 5A, showing the effect of adding BCAAs to the medium in the WT strain. C- Quantification (as relative amounts of the level observed in the absence of BCAAs) is shown only for the  $\Delta$ att strain.

### ***ilvL* translation mediates a BCAA-dependent inhibition of transcription through transcriptional attenuation.**

We questioned the functionality of the *ilvL* transcription attenuator and the requirement of *ilvL* translation for regulation in *V. alginolyticus*. The chromosomal endogenous *ilvL* locus was replaced by the *ilvL* $\Delta$ att and the *ilvL* $\Delta$ ATG mutations described above, and the expression of the *IlvGMEDA* operon was monitored in these mutants (Fig. 5 & 6).

Firstly, the *ilvG* and *ilvA* transcript levels clearly showed a very strong increase of expression when the attenuator sequence had been removed. In addition, quantification of the transcripts relative to the level in absence of any BCAAs, shows that the presence of BCAAs in the environment could not

repress *ilvGMEDA* expression in absence of the attenuator (Fig. 5C), leading to the conclusion that its role is to block the downstream *ilv* genes expression in presence of BCAAs.

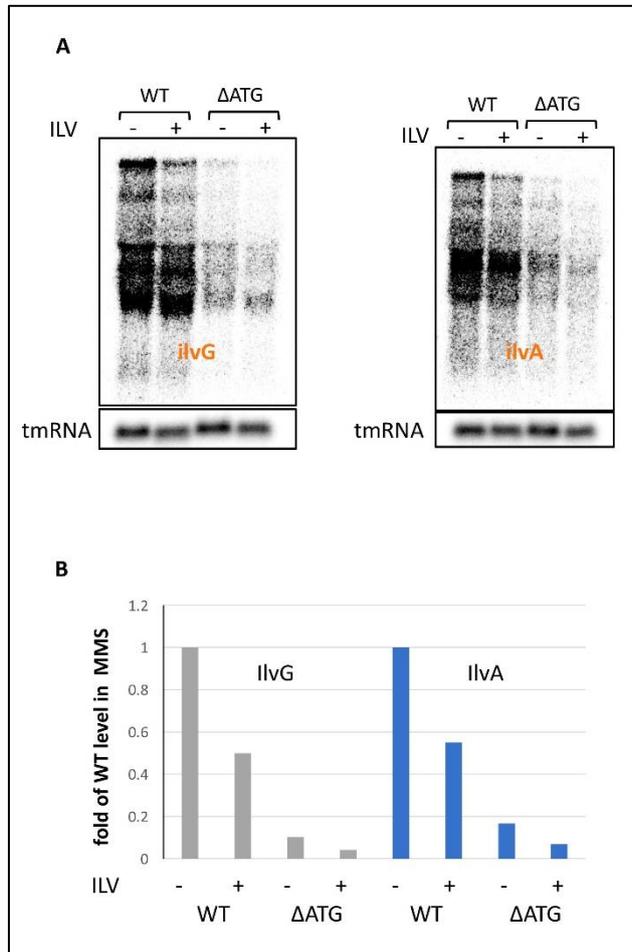


Fig. 6. IlvL translation is required for the transcription of the *ilvG* and *ilvA* genes. A- Northern blots in absence (-) or presence of all three BCAAs (+) of *ilvG* (left panel) or *ilvA* (right panel) in WT strain and the *ilvLΔATG* mutant. B-Quantification of northern blots of Fig. 6A. Expression is expressed as relative amounts of the level observed in the WT strain in the absence of BCAAs (-ILV). Grey histograms: IlvG probe. Blue histograms: IlvA probe.

On the contrary, when the initiation codon ATG was deleted from the *ilvL* ORF, a very strong reduction of either *ilvG* or *ilvA* was observed even in absence of BCAAs (Fig. 6), indicating that translation of *ilvL* is essential to overcome the action of the transcription terminator. However, in absence of translation of the leader peptide (*ilvLΔATG* strain), addition of ILV had still an inhibitory effect, suggesting that BCAAs could also control the expression of the *ilvGMEDA* through another mechanism than the modulation of transcription termination through *ilvL* translation. One way could have been via an effect at the transcriptional level, for instance mediated by Lrp. However, this seems to be excluded by the absence of effect on *ilvL* transcription of adding BCAAs (Fig. 4B & 4C). Alternatively, post-transcriptional regulation could be indirect, through the action of an sRNA

for instance. To our best of knowledge, no sRNA has been described that regulates the biosynthesis of branched-chain amino-acids. This was not explored further.

We also determined the effect of these mutations on BCAAs auxotrophy. Deletion of the attenuator (Fig. 7, *ilvLΔatt* mutant) did not impair the ability of the strain to grow in absence

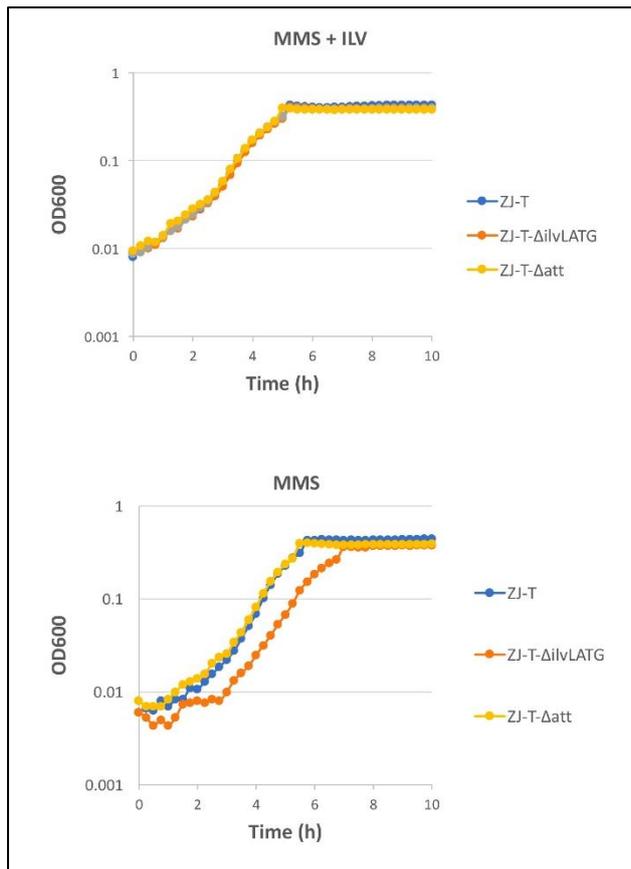


Fig. 7. *IlvL* translation is required for optimal biosynthesis of BCAAs. Cells from various mutant strains as indicated on the right were grown in MMS (lower panel) and MMS supplemented by BCAAs (upper panel).

of BCAAs, as expected since in this strain the expression of *ilvGMEDA* is constitutive. In contrast, the *ilvLΔATG* mutant showed a significant decrease of growth rate in absence of these AAs, reflecting the decreased biosynthetic activity of the strain due to increased transcription termination in absence of *ilvL* translation.

### Conservation of the mechanism of transcription attenuation between Enterobacteriaceae and Vibrionaceae

In Gram-negative bacteria, transcriptional attenuation has been studied mostly in *E. coli* and *Salmonella sp.* (Gollnick & Babitzke, 2002), two Enterobacteriaceae belonging to the class of  $\gamma$ -proteobacteria.

A genomic study of proteobacteria pre-

dicted the presence of an attenuator in front of the *leu* and the *ilvGMEDA* operons in vibrios, suggesting a conserved mechanism of regulation of BCAA biosynthesis between Vibrionaceae and Enterobacteriaceae (Vitreschak et al, 2004). As said above, the 5'UTR region of *ilvGMEDA* operon in *V. alginolyticus* does not show any primary sequence similarity to the analogous region in *E. coli*, whereas in the *IlvL* ORF, only the BCAA codons are conserved. However, when analyzing the putative secondary structure of the *ilvL* transcript by Mfold (Zuker, 2003), we could identify all the features that have been described for the canonical *E. coli trp* leader transcript (Fig. 8) (reviewed in (Gollnick & Babitzke, 2002)). A first secondary structure (1:2) can form after the RNAP has initiated transcription, which acts as a pause site for transcription. This pause in transcription allows loading of the ribosome, which starts to translate the *ilvL*

ORF and disrupts the pause site. Transcription resumes, with translation coupled to transcription. In presence of sufficient amounts of Ile, Val and Leu, translation can proceed to the stop codon, where the ribosome is released. In this case, a Rho independent terminator (structure 3:4) can form, preventing transcription of the downstream genes. On the contrary, in presence of low amounts of Ile, Val or Leu, ribosomes cannot proceed efficiently with the peptide translation, due to the lack of charged tRNAs, and remain stuck on the ILV codon-rich regions. This prevents the formation of a transcription pause site, and allows the base-pairing of RNA sequences 2 with 3, forming an alternative anti transcription termination structure. Ribosome stalling, by preventing the formation of a terminator, allows the RNA polymerase to transcribe the *ilvGMEDA* genes.

How well is such mechanism conserved in the *Vibrio* genus? A *blastn* search using the *V. alginolyticus ilvL* mRNA sequence as a query did not retrieve similar sequences beyond the *V. harveyi* clade, to which belongs *V. alginolyticus* (Sawabe et al, 2013b). Especially, no hits were obtained in *V. cholerae* genomes. However, the alignment of the upstream region of *ilvG* in the two species showed a strong conservation of features characteristic of transcription attenuation: the presence of an ORF rich in codons for BCAAs followed by a highly conserved attenuator structure, suggesting that all vibrios share this mechanism to regulate BCAA biosynthesis (Fig. 9).

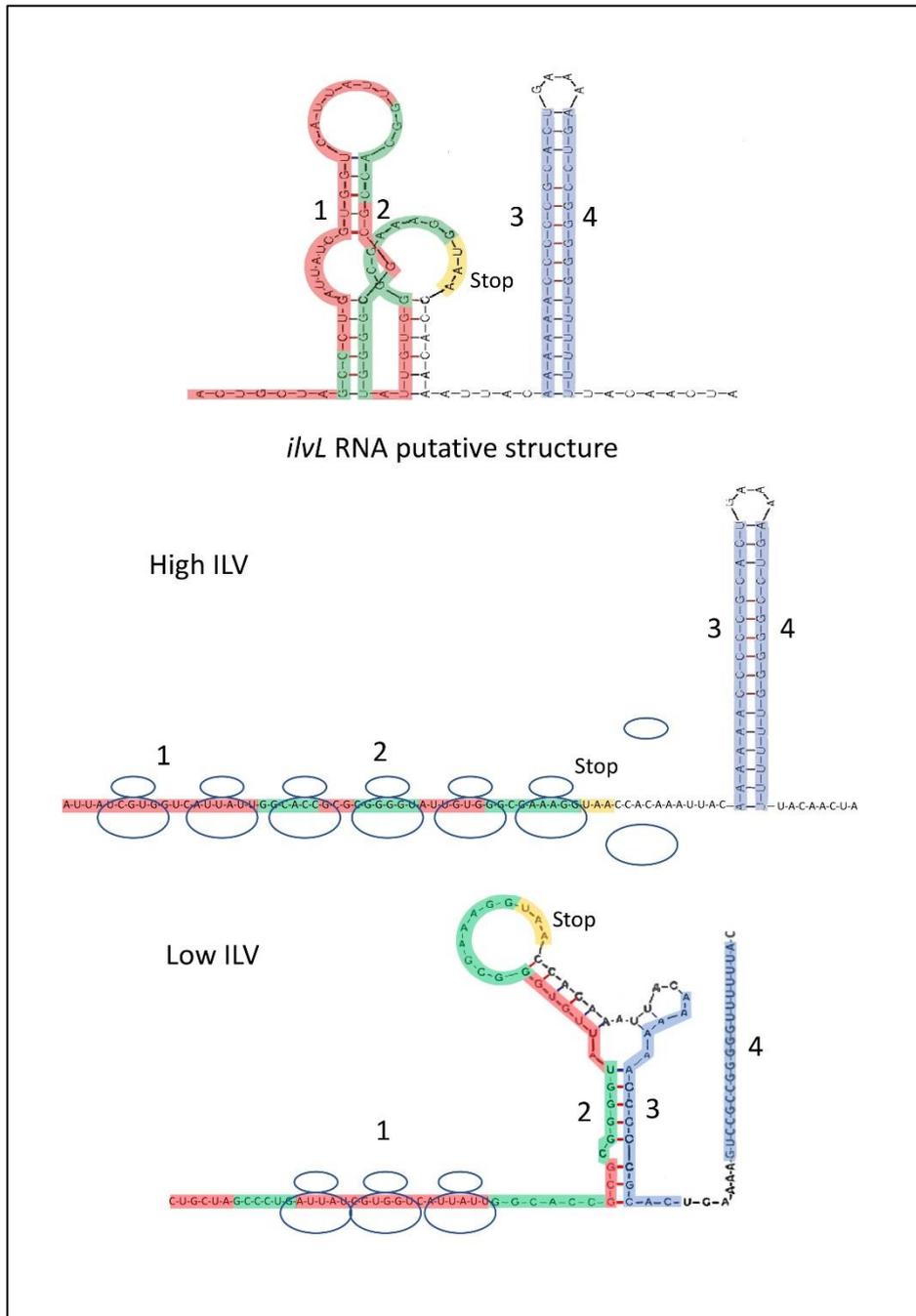


Fig. 8. A model for transcription attenuation of the *ilvGMEDA* operon in *V. alginolyticus*. Top : *ilvL* RNA putative secondary structure was calculated using Mfold (version 2.3; <http://unafold.rna.albany.edu/?q=mfold>)(Zuker, 2003) setting up the folding temperature at 30°C. The *ilvL* ORF is highlighted: red; BCAA codons, green; other codons, yellow; stop codon. The putative attenuator is highlighted in blue. Regions involved in secondary structures are numbered 1-4. Middle: In presence of high BCAA (ILV) concentration, ribosomes can proceed with translation until the stop codon where they are released. Region 2 is translated, allowing regions 3 and 4 to hybridize and form the attenuator. The downstream genes are not transcribed. Bottom: when BCAA concentration is low, ribosomes reached the BCAA codon rich region and cannot proceed because of a lack of charged tRNAs. Region 2 is now free to hybridize with region 3, forming an alternative

antitermination structure, and preventing the formation of the terminator. See text for more details on this model.

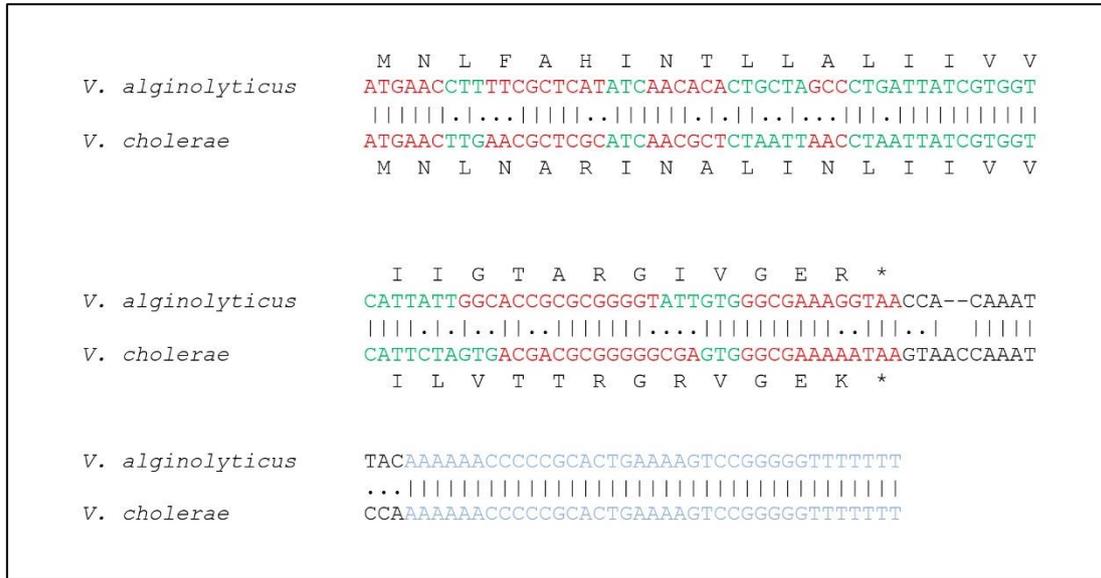


Fig. 9. Structural conservation of the *ilvG* upstream region in *V. alginolyticus* ZJ-T and *V. cholerae*. The upstream regions of *ilvG* from *V. alginolyticus* ZJ-T and *V. cholerae* N16961 were aligned using EMBOSS Needle ([https://www.ebi.ac.uk/Tools/psa/emboss\\_needle/](https://www.ebi.ac.uk/Tools/psa/emboss_needle/)). The BCAA codons are colored in green, other codons in red, and the putative attenuator structure is in blue.

## Conclusion

Transcription attenuation is thought to be a widely used mechanism for gene regulation in bacteria. In Gram-negative bacteria, transcription attenuation of amino-acid biosynthetic operons has been experimentally studied mostly in enteric bacteria such as *E. coli* and *Salmonella sp.* (Gollnick & Babitzke, 2002) and so far, to our best of knowledge, no experimental studies have been reported for other proteobacteria species. More recent studies focused on Gram-positive bacteria such as *B. subtilis* and *S. aureus*, showing that, although transcription attenuation does exist, global transcriptional regulators such as CodY and TnrA play an essential role (Kaiser et al, 2018; Tojo et al, 2005; Tojo et al, 2004). In the case of regulation of the ILV genes, genomic studies have predicted the existence of attenuators linked to leader peptides in  $\gamma$ ,  $\alpha$ , and  $\beta$  proteobacteria as well as in some low-GC Gram-positive bacteria (i.e *B. subtilis* and *S. aureus*)(Vitreschak et al, 2004). In the  $\gamma$ -proteobacteria, attenuators were predicted in front of

the BCAA operons *ilvGMEDA* and *leuABCD* in Enterobacteriaceae (with a few exceptions for *ilvGMEDA*) and in the three vibrios which were examined, suggesting that a similar mechanism was operating in the *Vibrio* genus (Vitreschak et al, 2004). Our results confirm experimentally that this is indeed the case, illustrating a high degree of structural and mechanistic conservation beyond the lack of primary sequence conservation.

## Materials and methods

### Growth of bacterial strains

*V. alginolyticus* ZJ-T, a translucent variant of ZJ51 (Deng et al, 2016a) was used in this study preferentially to the original strain because its genome has been fully sequenced (ACC # CP016224 and CP016225). ZJ-T was cultured in LBS (Luria–Bertani with 3% NaCl) at 30°C, supplemented if needed by chloramphenicol (Cm, 5 µg.mL<sup>-1</sup>). *E. coli* strains were cultured in Luria–Bertani (LB) medium at 37°C or M63 medium (3 g.L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 7 g.L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 2 g.L<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5×10<sup>-3</sup> g.L<sup>-1</sup> FeSO<sub>4</sub>, 2×10<sup>-3</sup> M MgSO<sub>4</sub>) containing 10g.L<sup>-1</sup> NaCl and supplemented with 4 g.L<sup>-1</sup> D-glucose and 5×10<sup>-3</sup> g.L<sup>-1</sup> thiamine. If needed, Cm was added at 20 µg.mL<sup>-1</sup>. For transconjugants selection, TCBS medium (Becton Dickinson, USA) + Cm (5 µg.mL<sup>-1</sup>) was used. To select transconjugants having undergone plasmid excision and allelic exchange, expression of the *ccdB* toxin encoding gene carried by the pSW7848 suicide plasmid (Val et al, 2012) was induced by adding 0.2% L-arabinose to the medium. Minimal medium with salt (MMS), used for *V. alginolyticus*, is a M63 based medium containing 30g.L<sup>-1</sup> NaCl and supplemented by 4 g.L<sup>-1</sup> D-glucose and 5×10<sup>-3</sup> g.L<sup>-1</sup> thiamine. When indicated, BCAAs were added at a final concentration of 20 mM each. Bacterial strains used in this study are listed in Table 1.

### Measurement of bacterial growth

*V. alginolyticus* strains were grown overnight in LBS medium at 30°C with shaking at 200 rpm. Overnight cultures were collected by centrifugation, washed twice with MMS, and resuspended in the same medium to an OD<sub>600nm</sub> (OD600) ≈ 0.05. Cultures (3 replicates in each case) were then incubated in a microplate reader (EnSpire, Australia) at 30°C with shaking at 200 rpm in 96 well plates. OD600 was measured at regular time intervals as indicated.

### **5' and 3' rapid amplification of cDNA ends (RACE)**

For 5' and 3' RACE, an O/N culture in LBS of the strain ZJ-T was washed twice with MMS before being resuspended at OD<sub>600</sub> ≈ 0.01 in either MMS or MMS+ILV. The bacterial cells were collected after 3 h, 6 h and 9 h of growth at 30°C. Total RNA was isolated using RNAPrep Pure Cell/Bacteria Kit (TIANGEN, China) and for each culture, RNA preparations from the different time points were pooled together. 5' and 3' RACE experiments were then carried out using SMARTer® RACE 5'/3' Kit (Clontech, USA) according to the manufacturer's instructions. At least 12 resulting clones were sequenced to determine the ends of the *ilvL* and the *ilvG* transcripts.

### **Plasmid construction and gene disruption**

*V. alginolyticus* ZJ-T in-frame deletion mutants were constructed as described previously (Nguyen et al, 2018a). Briefly, amplified linearized pSW7848 plasmid DNA (Val et al, 2012) was assembled by isothermal assembly using ClonExpress MultiS One Step Cloning Kit (Vazyme, China) with purified PCR products corresponding to the flanking regions of the desired deletion, and introduced into *E. coli* II3813 (Le Roux et al, 2007) as an intermediate cloning strain, selecting recombinant cells on 20 µg/ml Cm and 0.3 mM thymidine. The recombinant plasmid was then introduced into the donor strain GEB883 (Nguyen et al, 2018a) (Table 1) for transfer by conjugation to *V. alginolyticus* ZJ-T. pSW7848 requires the Pir<sup>+</sup> protein for its replication which is present in the donor strain but absent in vibrios where the suicide plasmid inserts at the targeted locus by homologous recombination, generating a duplication. Plating out the exconjugants on arabinose induces expression of the *ccdB* gene, present on pSW7848 and select for bacteria having lost the inserted plasmid, and one copy of the targeted locus. The presence of the mutation was then confirmed by PCR amplification and sequencing. Transcriptional fusions to GFP were constructed as above by assembling the region to be assayed for promoter activity with the *gfp* gene amplified from pJBA113 (Andersen et al, 1998) and a plasmid template derived from pSCT32 (Zhang et al, 2017b). The Gfp encoded by pJBA113 has a half-life of approximately 110 min in *E. coli* (Andersen et al, 1998). All plasmids are described in Table 1. All oligonucleotides used in this study are listed on Table 2.

## Gfp measurement

*E. coli* BL21 was transformed by the plasmids containing the *gfp* fusions to be assayed, and Gfp activity was measured as followed: cells from overnight cultures in LB supplemented by Cm were washed twice in M63 medium containing 10 g.L<sup>-1</sup> NaCl and D-glucose as a carbon source and resuspended to OD600≈0.05 in the same medium with 20 μg.mL<sup>-1</sup> Cm. The diluted cultures were added to 96 well plates (100 μL/well) and incubated in a microplate reader (En-Spire, Australia) at 37 °C with shaking. OD600 and Gfp fluorescence (FU) (excitation 485nm/emission 535 nm) were monitored during growth. Background fluorescence measured with strain BL21/pSCT32-*gfp* (with no insert upstream of the promoter-less *gfp*) was deducted from the measured FU. Relative fluorescence unit (RFU = FU/OD600) was then calculated for each time point.

## Northern blotting

*V. alginolyticus* WT and mutant cells were cultured overnight in MMS, diluted to OD600 ≈ 0.05 in the same medium and grown at 30°C with continuous shaking at 180 rpm. When OD600 reached 0.3, cells were centrifuged and resuspended at the same OD600 in MMS supplemented or not, as required, by all three BCAAs or any one of them as indicated. After 30 min of further incubation, bacterial cells were collected for total RNA extraction, which was carried out using Direct-zol MiniPrep Plus (Zymo research). After DNase treatment, 10 μg of purified RNA was used for Northern blotting as described previously (Toffano-Nioche et al, 2012). Oligonucleotide probes complementary to the gene to be assayed (Table 2) were labeled at their 3' end using terminal transferase (Fermentas, USA) and [ $\alpha$ -32p] dCTP. RiboRuler Low and High Range RNA Ladder (Fermentas, USA) were run alongside the samples to allow an estimation of the transcript size. tmRNA was used as a loading control. Transcripts were quantified by image J.

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## References

1. Salmon K, Yang C, Hatfield G. 2006. Biosynthesis and Regulation of the Branched-Chain Amino Acids†. EcoSal Plus doi:doi:10.1128/ecosalplus.3.6.1.5.
2. Willins DA, Calvo JM. 1992. In vitro transcription from the *Escherichia coli ilvIH* promoter. J Bacteriol 174:7648-55.
3. Wek RC, Hatfield GW. 1986. Nucleotide sequence and in vivo expression of the *ilvY* and *ilvC* genes in *Escherichia coli* K12. Transcription from divergent overlapping promoters. J Biol Chem 261:2441-50.
4. Newman EB, Lin R. 1995. Leucine-responsive regulatory protein: a global regulator of gene expression in *E. coli*. Annu Rev Microbiol 49:747-75.
5. Searles LL, Wessler SR, Calvo JM. 1983. Transcription attenuation is the major mechanism by which the *leu* operon of *Salmonella typhimurium* is controlled. J Mol Biol 163:377-94.
6. Wessler SR, Calvo JM. 1981. Control of *leu* operon expression in *Escherichia coli* by a transcription attenuation mechanism. J Mol Biol 149:579-97.
7. Hauser CA, Hatfield GW. 1983. Nucleotide sequence of the *ilvB* multivalent attenuator region of *Escherichia coli* K12. Nucleic Acids Res 11:127-39.
8. Lawther RP, Wek RC, Lopes JM, Pereira R, Taillon BE, Hatfield GW. 1987. The complete nucleotide sequence of the *ilvGMEDA* operon of *Escherichia coli* K-12. Nucleic Acids Res 15:2137-55.
9. Hauser CA, Hatfield GW. 1984. Attenuation of the *ilvB* operon by amino acids reflecting substrates or products of the *ilvB* gene product. Proc Natl Acad Sci U S A 81:76-9.
10. Nargang FE, Subrahmanyam CS, Umbarger HE. 1980. Nucleotide sequence of *ilvGEDA* operon attenuator region of *Escherichia coli*. Proc Natl Acad Sci U S A 77:1823-7.
11. Brinsmade SR. 2017. CodY, a master integrator of metabolism and virulence in Gram-positive bacteria. Curr Genet 63:417-425.
12. Guedon E, Serror P, Ehrlich SD, Renault P, Delorme C. 2001. Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. Mol Microbiol 40:1227-39.
13. Kaiser JC, King AN, Grigg JC, Sheldon JR, Edgell DR, Murphy MEP, Brinsmade SR, Heinrichs DE. 2018. Repression of branched-chain amino acid synthesis in *Staphylococcus aureus* is mediated by isoleucine via CodY, and by a leucine-rich attenuator peptide. PLoS Genet 14:e1007159.

14. Vitreschak AG, Lyubetskaya EV, Shirshin MA, Gelfand MS, Lyubetsky VA. 2004. Attenuation regulation of amino acid biosynthetic operons in proteobacteria: comparative genomics analysis. *FEMS Microbiol Lett* 234:357-70.
15. Lintner RE, Mishra PK, Srivastava P, Martinez-Vaz BM, Khodursky AB, Blumenthal RM. 2008. Limited functional conservation of a global regulator among related bacterial genera: Lrp in *Escherichia*, *Proteus* and *Vibrio*. *BMC Microbiol* 8:60.
16. Citil BE, Derin S, Sankur F, Sahan M, Citil MU. 2015. *Vibrio alginolyticus* Associated chronic coryngitis acquired in Mediterranean waters of Turkey. *Case Rep Infect Dis* 2015:187212.
17. Siddiqui R, Alam MM, Naser MN, Otomo Y, Yasmin M, Nessa J, Ahsan CR. 2016. Prevalence of *Vibrio alginolyticus* in sediment samples of river and coastal areas of Bangladesh. 29:1-6.
18. Wek RC, Hauser CA, Hatfield GW. 1985. The nucleotide sequence of the *ilvBN* operon of *Escherichia coli*: sequence homologies of the acetohydroxy acid synthase isozymes. *Nucleic Acids Research* 13:3995-4010.
19. Baccigalupi L, Marasco R, Ricca E, De Felice M, Sacco M. 1995. Control of *ilvIH* transcription during amino acid downshift in stringent and relaxed strains of *Escherichia coli*. *FEMS Microbiol Lett* 131:95-8.
20. Levinthal M, Lejeune P, Danchin A. 1994. The H-NS protein modulates the activation of the *ilvIH* operon of *Escherichia coli* K12 by Lrp, the leucine regulatory protein. *Mol Gen Genet* 242:736-43.
21. Friden P, Newman T, Freundlich M. 1982. Nucleotide sequence of the *ilvB* promoter-regulatory region: a biosynthetic operon controlled by attenuation and cyclic AMP. *Proc Natl Acad Sci U S A* 79:6156-60.
22. Gollnick P, Babitzke P. 2002. Transcription attenuation. *Biochim Biophys Acta* 1577:240-50.
23. Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31:3406-15.
24. Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AR, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T. 2013. Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. *Front Microbiol* 4:414.
25. Tojo S, Satomura T, Morisaki K, Deutscher J, Hirooka K, Fujita Y. 2005. Elaborate transcription regulation of the *Bacillus subtilis ilv-leu* operon involved in the biosynthesis of branched-chain amino acids through global regulators of CcpA, CodY and TnrA. *Molecular Microbiology* 56:1560-1573.
26. Tojo S, Satomura T, Morisaki K, Yoshida K-I, Hirooka K, Fujita Y. 2004. Negative Transcriptional regulation of the *ilv-leu* operon for biosynthesis of branched-chain amino acids through the *Bacillus subtilis* global regulator TnrA. *Journal of Bacteriology* 186:7971-7979.
27. Deng Y, Chen C, Zhao Z, Huang X, Yang Y, Ding X. 2016. Complete genome sequence of *Vibrio alginolyticus* ZJ-T. *Genome Announc* 4(5). pii: e00912-16. doi: 10.1128/genomeA.00912-16.
28. Val ME, Skovgaard O, Ducos-Galand M, Bland MJ, Mazel D. 2012. Genome engineering in *Vibrio cholerae*: a feasible approach to address biological issues. *PLoS Genet* 8:e1002472.

29. Nguyen AN, Disconzi E, Charriere GM, Destoumieux-Garzon D, Bouloc P, Le Roux F, Jacq A. 2018. *csrB* Gene duplication drives the evolution of redundant regulatory pathways controlling expression of the major toxic secreted metalloproteases in *Vibrio tasmaniensis* LGP32. *mSphere* 3(6). pii: e00582-18. doi: 10.1128/mSphere.00582-18.
30. Le Roux F, Binesse J, Saulnier D, Mazel D. 2007. Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl Environ Microbiol* 73:777-84.
31. Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M, Molin S. 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* 64:2240-6.
32. Zhang YJ, Chen G, Lin H, Wang P, Kuang B, Liu J, Chen S. 2017. Development of a regulatable expression system for the functional study of *Vibrio vulnificus* essential genes. *Antonie Van Leeuwenhoek* 110:607-614.
33. Toffano-Nioche C, Nguyen AN, Kuchly C, Ott A, Gautheret D, Bouloc P, Jacq A. 2012. Transcriptomic profiling of the oyster pathogen *Vibrio splendidus* opens a window on the evolutionary dynamics of the small RNA repertoire in the *Vibrio* genus. *RNA* 18:2201-19.
34. Xie ZY, Hu CQ, Chen C, Zhang LP, Ren CH. 2005. Investigation of seven *Vibrio* virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong, China. *Lett Appl Microbiol* 41:202-7.
35. Chang C, Jin X, Chaoqun H. 2009. Phenotypic and genetic differences between opaque and translucent colonies of *Vibrio alginolyticus*. *Biofouling* 25:525-531.

**Table 1. Strains and plasmids used in this study.**

Strain or plasmid	Relevant characteristics	Source
<b><i>V. alginolyticus</i></b>		
ZJ-T	Ap <sup>r</sup> (ampicillin resistant), translucent/smooth variant of wild strain ZJ51 (Xie et al, 2005); isolated from diseased <i>Epinephelus coioides</i> off the Southern China coast	(Chang et al, 2009)
ZJ-T- <i>ilvLATG</i>	<i>ilvL</i> Δ <i>ATG</i>	This study
ZJ-T-Δ <i>ilvL</i> Δ <i>att</i>	<i>ilvL</i> Δattenuator	This study
ZJ-T-Δ <i>ilvIH</i>	ZJ-T carrying a deletion of <i>ilvIH</i>	This study
ZJ-T-Δ <i>ilvB</i>	ZJ-T carrying a deletion of <i>ilvB</i>	This study
ZJ-T-Δ <i>ilvGM</i>	ZJ-T carrying a deletion of <i>ilvGM</i>	This study
ZJ-T-Δ <i>ilvIH-B</i>	ZJ-T carrying deletions of <i>ilvIH</i> and <i>ilvB</i>	This study
ZJ-T-Δ <i>ilvIH-GM</i>	ZJ-T carrying deletions of <i>ilvIH</i> and <i>ilvGM</i>	This study
ZJ-T-Δ <i>ilvB-GM</i>	ZJ-T carrying deletions of <i>ilvB</i> and <i>ilvGM</i>	This study
<b><i>E. coli</i></b>		
Π3813	<i>E. coli</i> K12 <i>lacIQ</i> , <i>thi1</i> , <i>supE44</i> , <i>endA1</i> , <i>recA1</i> , <i>hsdR17</i> , <i>gyrA462</i> , <i>zei298::tn10[Tc]</i> , <i>ΔthyA::erm-pir116[Er]</i> ; cloning host	(Le Roux et al, 2007)
GEB883	<i>E. coli</i> K12 Δ <i>dapA::erm pir[Er]</i> RP4-2 Δ <i>recA</i> <i>gyrA462</i> , <i>zei298::tn10[Tc]</i> ; donor strain for conjugation	(Nguyen et al, 2018a)
BL21(DE3)	F <sup>-</sup> <i>ompT hsdSB (rB mB)</i> <i>gal dcm</i> (DE3)	Laboratory collection
<b>Plasmids</b>		
pSCT32-[-147, +1]:: <i>gfp</i>	Cm <sup>r</sup> ; transcriptional fusion [-147 +1]:: <i>gfp</i>	This study
pSCT32-[-147, -53]:: <i>gfp</i>	Cm <sup>r</sup> ; transcriptional fusion [-147 -53]:: <i>gfp</i>	This study
pSCT32-[-53, +1]:: <i>gfp</i>	Cm <sup>r</sup> ; transcriptional fusion [-53 +1]:: <i>gfp</i>	This study
pSCT32-[-147, +303]:: <i>gfp</i>	Cm <sup>r</sup> ; transcriptional fusion [-147+303]:: <i>gfp</i>	This study
pSCT32-[-147, +303]:: <i>gfp</i> Δ <i>ilvLATG</i>	Cm <sup>r</sup> ; transcriptional fusion [-147+303 Δ <i>ilvLATG</i> ]:: <i>gfp</i>	This study

pSCT32-[-147, +303]:: <i>gfp</i> $\Delta$ <i>ilvLatt</i>	Cm <sup>r</sup> ; transcriptional fusion [-147+303 $\Delta$ <i>ilvLatt</i> ]:: <i>gfp</i>	This study
pSCT32- <i>gfp</i>	Cm <sup>r</sup> ; <i>gfp</i> fusion vector	This study
pSCT32	Cm <sup>r</sup> ; pBR322 origin	(Zhang et al, 2017b)
pSW7848	Cm <sup>r</sup> ; suicide vector with an R6K origin, requiring the Pir protein for its replication, pBAD- <i>ccdB</i>	(Val et al, 2012)
pSW7848- $\Delta$ <i>ilvLATG</i>	Cm <sup>r</sup> ; pSW7848 carrying the mutant allele <i>ilvLATG</i>	This study
pSW7848- $\Delta$ <i>ilvLatt</i>	Cm <sup>r</sup> ; pSW7848 carrying the mutant allele <i>ilvLatt</i>	This study
pSW7848- $\Delta$ <i>ilvIH</i>	Cm <sup>r</sup> ; pSW7848 carrying the <i>ilvIH</i> deletion	This study
pSW7848- $\Delta$ <i>ilvB</i>	Cm <sup>r</sup> ; pSW7848 carrying the <i>ilvB</i> deletion	This study
pSW7848- $\Delta$ <i>ilvGM</i>	Cm <sup>r</sup> ; pSW7848 carrying the <i>ilvGM</i> deletion	This study

**Table 2. Nucleotide sequences of primers and probes used in this study**

Non-target bases complementary to the cloning vector are indicated by lower case letters

Name	Sequence (5'-3')	Targets/Goal
<b>Northern blot probes</b>		
NB- <i>ilvL</i>	AATGACCACGA- TAATCAGGGCTAGCAG	<i>ilvL</i>
NB- <i>ilvG</i>	GGTACCGAGGCCTTTTAATGTGCTAAC	<i>ilvG</i>
NB- <i>ilvA</i>	CTTGATGCCTAGCTTAGTACCAGAG	<i>ilvA</i>
NB- <i>ilvI</i>	CCGAAATAACGATCATTGGGATGG	<i>ilvI</i>
NB-tm RNA	GGAAGCTAGGGCGAGAGAGCTCTTAG	tmRNA
<b>5' and 3' RACE</b>		
RACE-UPML	TAATACGACTCACTATAGGGCAAGCAGTGG- TATCAACGCAGAGT	RACE product cloning
RACE-UPMS	CTAATACGACTCACTATAGGGC	RACE product cloning
RACE- <i>ilvL</i> -F	gattacccaagcttACAGACAGATGAACCTTTTCGCTC	<i>ilvL</i> transcript
RACE- <i>ilvL</i> -R	gattacccaagcttGCGCGGTGCCAATAATGACCA	<i>ilvL</i> transcript
RACE- <i>ilvG</i> -R	gattacccaagcttGGGTAGCCAAAACGGTTTCG	<i>ilvG</i> transcript
<b><i>gfp</i>- transcriptional fusion</b>		
pSCT32-GFP-TR-F	AGAATTAAGAGGAGAAATTAAGCATGC	<i>gfp</i>
pSCT32-GFP-TR-R	AGATAGATCTTGCATGCGGG	<i>gfp</i>
[-147+1]::GFP-TR-F	gcatgcaagatctatctACGTTCCCTCAATTTTTTT- GAACACTC	[-147, +1]
[-147+1]::GFP-TR-R	atttctcctttaattctCGTCATTAGTGGTATCACACAATT	[-147, +1]
[-147-53]::GFP-TR-F	gcatgcaagatctatctACGTTCCCTCAATTTTTTTGAACAC	[-147, +53]
[-147-53]::GFP-TR-R	atttctcctttaattctTTTTTCACATTA ACTCTCAATT- GGGA	[-147, +53]
[-53+1]::GFP-TR-F	gcatgcaagatctatctAAGAATGACTTTTTGCTTGTCATG	[-53,+1]
[-53+1]::GFP-TR-R	atttctcctttaattctCGTCATTAGTGGTATCACACAATT	[-53,+1]
[-147+303]::GFP-TR-F	gcatgcaagatctatctACGTTCCCTCAATTTTTTT- GAACACTC	[-147,+303], <i>WT, ilvLΔATG,</i> <i>ilvLΔatt</i>
[-147+303]::GFP-TR-R	atttctcctttaattctCTACGTTGTGGCTCGTAGCG	[-147,+303], <i>WT, ilvLΔATG,</i> <i>ilvLΔatt</i>
pSCT32::GFP-check-F	TGATGCCGCATAGTTAAGCCAG	verification
pSCT32::GFP-check-R	CTTGGAGCCGTACTGGA ACTGA	verification
<b>Mutagenesis</b>		
pSW7848-F	GTCTGATTCGTTACCAATTATGACAAC	vector
pSW7848-R	GAATTCGATATCAAGCTTATCGATAC	vector
Del-check-pSW7848-F	TCACTGTCCCTTATTCGCACC	verification

Del-check-pSW7848-R ilvLATG-UP-F	CTGCTTTTGAGCACTACCCG aagcttgatatcgaattcTGTTAGATAGTCTAGAGTGATGGG	verification <i>ilvLΔATG</i>
ilvLATG-UP-R	gcgaaaaggttCTGTCTGTCGTTCTTTTTTC	<i>ilvLΔATG</i>
ilvLATG-DOWN-F	acagacagAACCTTTTCGCTCATATCAACAC	<i>ilvLΔATG</i>
ilvLATG-DOWN-R	ttgtaacgaatcagacGGTGCCATGCATACCCAAC	<i>ilvLΔATG</i>
ilvLatt-UP-F	aagcttgatatcgaattcGAACTCATTAAGATTAGTGTTCAA- TATTG	<i>ilvLΔatt</i>
ilvLatt-UP-R	aactgttagTTTGTGGTTACCTTTCGCCC	<i>ilvLΔatt</i>
ilvLatt-DOWN-F	aaccacaaaCTACAAGTTGTCATCTGTCTAACC	<i>ilvLΔatt</i>
ilvLatt-DOWN-R	ttgtaacgaatcagacGTGGCGCAGTTTATGAATCTC	<i>ilvLΔatt</i>
ilvB-UP_fwd	aagcttgatatcgaattcGCCATCGCTCGGGTAAGG	$\Delta ilvB$
ilvB-UP_rev	tcaatgaattTACTTAAGCTGTTTTTCGAGCTCG	$\Delta ilvB$
ilvB-DOWN_fwd	gcttaagtaAATTCATTGAACGTACCTAAAC- CAAATCCC	$\Delta ilvB$
ilvB-DOWN_rev	ttgtaacgaatcagacGCGCCATGGCAATCGTCG	$\Delta ilvB$
ilvIH-UP_fwd	aagcttgatatcgaattcTGCGTGGCTATTACAAAATG	$\Delta ilvIH$
ilvIH-UP_rev	gcagtgacAATTGCTCTCCTTAGCCAG	$\Delta ilvIH$
ilvIH-DOWN_fwd	agagcaattGTCACTGCTGCTCAATACACG	$\Delta ilvIH$
ilvIH-DOWN_rev	ttgtaacgaatcagacGCCAAATGTTGGGGTACATC	$\Delta ilvIH$
ilvGM-UP_fwd	aagcttgatatcgaattcGCGTTTGAAAAAGATTTCAG	$\Delta ilvGM$
ilvGM-UP_rev	ctactttgcCGTAAACCTCCTTTGCTAC	$\Delta ilvGM$
ilvGM-DOWN_fwd	ggtttacgGCAAAGTAGCCAGCGTCG	$\Delta ilvGM$
ilvGM-DOWN_rev	ttgtaacgaatcagacCCAGTGCCCACTTCAATC	$\Delta ilvGM$
del ilvLATG-check-F	GTTATGTTATATACCGCGTCCC	verification
del ilvLATG-check-R	GACAGATGACAACTTGTAGTTGT	verification
del ilvLatt check-F	CGCAAACAATTTTCGTAGAACATTAC	verification
del ilvLatt check-R	GACAAGTTGTGCACCAGTCAT	verification
del-ilvB-check-F	GTTATCAACCCACACAATATGGAC	verification
del-ilvB-check-R	CTCTTCATTGCATAACTGGTCTTAC	verification
del-ilvIH-check-F	GTGGCTGCTATTCTACTAAC	verification
del-ilvIH-check-R	CGTCTACTGCCTATAATGCTTAC	verification
del-ilvGM-check-F	GTATTGTGGGCGAAAGGTAAC	verification
del-ilvGM-check-R	CTTCCTTATGCACTTACGTGTT	verification



## CHAPTER II

**A novel sRNA belonging to the maltose regulon and generated from the 5'UTR of *malK* in the *Vibrio* genus regulates the expression of a major enzyme of the glycolytic pathway**

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## Abstract

Vsr217 is a novel sRNA which was initially identified in the oyster pathogen *Vibrio tasmaniensis* LGP32, which has been associated with summer mortality events affecting juvenile oysters in France. In here, we show that it is conserved in the *Vibrio* genus where it is located upstream of *malK*, coding the ATPase component of the maltose importer. Vsr217 is induced by maltose and regulated by MalT, the positive regulator of the maltose regulon. We showed that Vsr217 is generated from the 5' UTR region of the *malK* message. Beside a cis-effect on *malK* expression, which decreases in the  $\Delta$ *vsr217* mutant, we found that the absence of this sRNA resulted, when the cells grown in maltose, in the increase of two important enzymes involved in the glycolysis/neoglucogenesis pathway, Fbp and PpsA and that *fbp* mRNA was a direct target of Vsr217. These two enzymes working only in the direction of neoglucogenesis, the induction of Vsr217 when the cells are growing in maltose would thus result in the stimulation of glycolysis.

## Keywords

5' UTR derived sRNA, maltose metabolism, neoglucogenesis, Fbp

## Introduction

In the last 15 years, the importance of non-coding RNAs (sRNAs) in bacterial regulatory networks has become widely recognized. The development of genome-wide transcriptomic studies, made possible by the remarkable progress of high-throughput sequencing techniques, has revealed a bounty of such potential regulators, much more than have been characterized in details so far. In addition, these methodologies can more easily discriminate between the different categories of sRNAs than previously used methods: *trans*-encoded or *cis*-encoded, and among the latter, *cis*-acting or *trans*-acting antisense RNAs.

They have also led to the exploration of the transcriptomic landscape of non-model bacterial organisms, pathogens or of environmental interest, leading to the discovery of many new potential sRNAs (for a review and specific examples see (Liu & Camilli; Miotto et al, 2012; van der Meulen et al, 2016), whereas the majority of well characterized sRNAs came from a relatively small number of bacterial species, mostly Enterobacteriaceae (*Escherichia coli* and *Salmonella* sp) and more recently, low GC gram positive bacteria such as *B. subtilis* or *Staphylococcus aureus* (Brantl & Bruckner, 2014; Wagner & Romby, 2015a).

A new class of sRNAs that have emerged from these most recent studies are sRNAs that are generated from the 5' and 3' untranslated regions (UTRs) of genes but that have distally encoded targets and act as *trans*-encoded sRNAs. For instance, in 2005, Kawano et al (Kawano et al, 2005), in a genome-wide search of small (<50 nt) regulatory RNAs in *E. coli* identified several ones corresponding to 5' or 3' UTRs. Several of such sRNAs have been characterized. For instance, CpxQ is generated from the 3' UTR of *cpxP* mRNA, a gene encoding a periplasmic chaperone involved in the inner membrane (IM) stress response. CpxQ was shown to repress several IM proteins, thus mitigating the IM stress (Chao & Vogel, 2016). Another example is MicL, which is transcribed from a  $\sigma^E$  dependent promoter internal to the upstream protein coding sequence, and then cleaved to a shorter product to act *in trans* to regulate expression of the major outer membrane lipoprotein Lpp (Guo et al, 2014a). *Trans*-acting sRNAs can also be generated from 5' UTRs. In the human pathogen *Listeria monocytogenes*, PrfA is a virulence

regulator whose expression is controlled by a thermosensor located in the 5' UTR of the *prfA* transcript (Johansson et al, 2002). (Loh et al, 2009) identified two S-adenosylmethionine (SAM) riboswitches SreA and SreB, which, like classical riboswitches, regulate their downstream gene expression, but also act *in trans* by targeting the 5' UTR of the *prfA* mRNA. Another recent example is the sRNA rnTrpL (Melior et al, 2019a) in *Sinorhizobium meliloti*, which is derived from a transcriptional attenuator of one of the three tryptophan operons and regulate *in trans* a second tryptophan operon which does not possess the leader peptide encoding attenuator. Other examples include Teg49 in *S. aureus*, derived from the 5' UTR of the virulence regulator gene *sarA* (Manna et al, 2018b), or *mals-5'UTR*, a *Salmonella enterica* ncRNA generated from the 5' UTR of *mals* that was proposed to regulate *hisG in trans*, a gene involved in the histidine biosynthetic pathway (Zhang et al, 2017a).

*Vibrio tasmaniensis* LGP32 (previously *V. splendidus* LGP32) is a member of the *V. splendidus* clade (Sawabe et al, 2013b) that has been associated with summer mortalities of juvenile Pacific oysters *Crassostrea gigas* (Gay et al, 2004b) and is a facultative intracellular pathogen of oysters, that invades host immune cells (Duperthuy et al, 2011; Vanhove et al, 2015; Vanhove et al, 2016). A genome-wide search for non-coding sRNAs in *V. tasmaniensis* LGP32 by RNA-seq led us to the identification of 250 potential trans-encoded sRNAs, among which several were confirmed by northern blots, and/or RT-PCR (Toffano-Nioche 2012). More recently, a transcriptomic analysis of LGP32 invading oyster hemocytes (the immune cells) revealed that, amongst these sRNAs, Vsr217 was one of the most highly induced gene (Vanhove et al, 2016). Additional qPCR experiments showed that this induction was not specific of the intrahemocyte condition but was also observed in the oyster hemolymph, compared to growth in sea water conditions (Vanhove et al, 2016). Interestingly, *vsr217* was located upstream of *malK*, a gene encoding the ATPase subunit of the maltose transporter, a member of the ABC family of transporter (reviewed in (Boos & Shuman, 1998). We also found that the maltose regulon was highly induced in oysters, a metabolic switch that can be related to the fact that oysters are extremely rich in glycogen, a precursor of maltose (Vanhove et al, 2016).

In here, we show that Vsr217 is part of the maltose regulon and is positively controlled by

the transcriptional regulator MalT. Most importantly, it is generated from the 5' UTR of *malK* and, beyond a positive role in *malK* expression, it acts as a trans-encoded sRNA to negatively control the expression of *fbp*, encoding a class I fructose-1,6-bisphosphatase, an enzyme involved in gluconeogenesis. Hence, *vsr217* is a new example of a growing class of trans-acting sRNAs that are derived from 5' or 3' UTRs.

## Results

### **The genetic organization of *Vsr217-malK* is conserved in other vibrios.**

To find out to which extent *Vsr217* was conserved in the *Vibrio* genus, we aligned *malK* upstream sequences from various vibrios representative of different *Vibrio* clades (Sawabe et al, 2013b) (Fig. 1). Results showed five regions of high conservation, interspaced with region of low or no conservation. The region I is immediately upstream of a -35 promoter sequence (P1) predicted by BPPROM (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>). The region II overlaps with the corresponding -10 motif of the same promoter and has similarity to a MalT box consensus sequence (5' -GGGGA(T/G)GAGG-3')(Vidal-Ingigliardi et al, 1991). MalT is the positive regulator of genes of the maltose regulon in *E. coli* and other Enterobacteriaceae (Schlegel et al, 2002). A third region corresponds to the 5' end of *Vsr217* as determined by 5'RACE (see below) and includes the 32 first nucleotides of the sRNA. Region IV contains a conserved motif TTTTC present at the 3' end of *vsr217*. Finally, the fifth region is the larger one and corresponds to the upstream region of the *malK* gene. This region contains a predicted sigma70 promoter (P2), as well as ribosome binding site for *malK*. Despite the conserved TTTTC motif present at the end of *Vsr217*, no rho independent terminator was identified between *vsr217* and *malK*.

In summary, this analysis shows that the regulatory regions of both *vsr217* and *malK*, as well as the 5' end and 3' end regions of the sRNA are conserved at the nucleotide sequence level in different vibrios, but that the overall sequence is not conserved. In addition, no rho independent terminator was predicted between the 3'end of *vsr217* and *malK*.

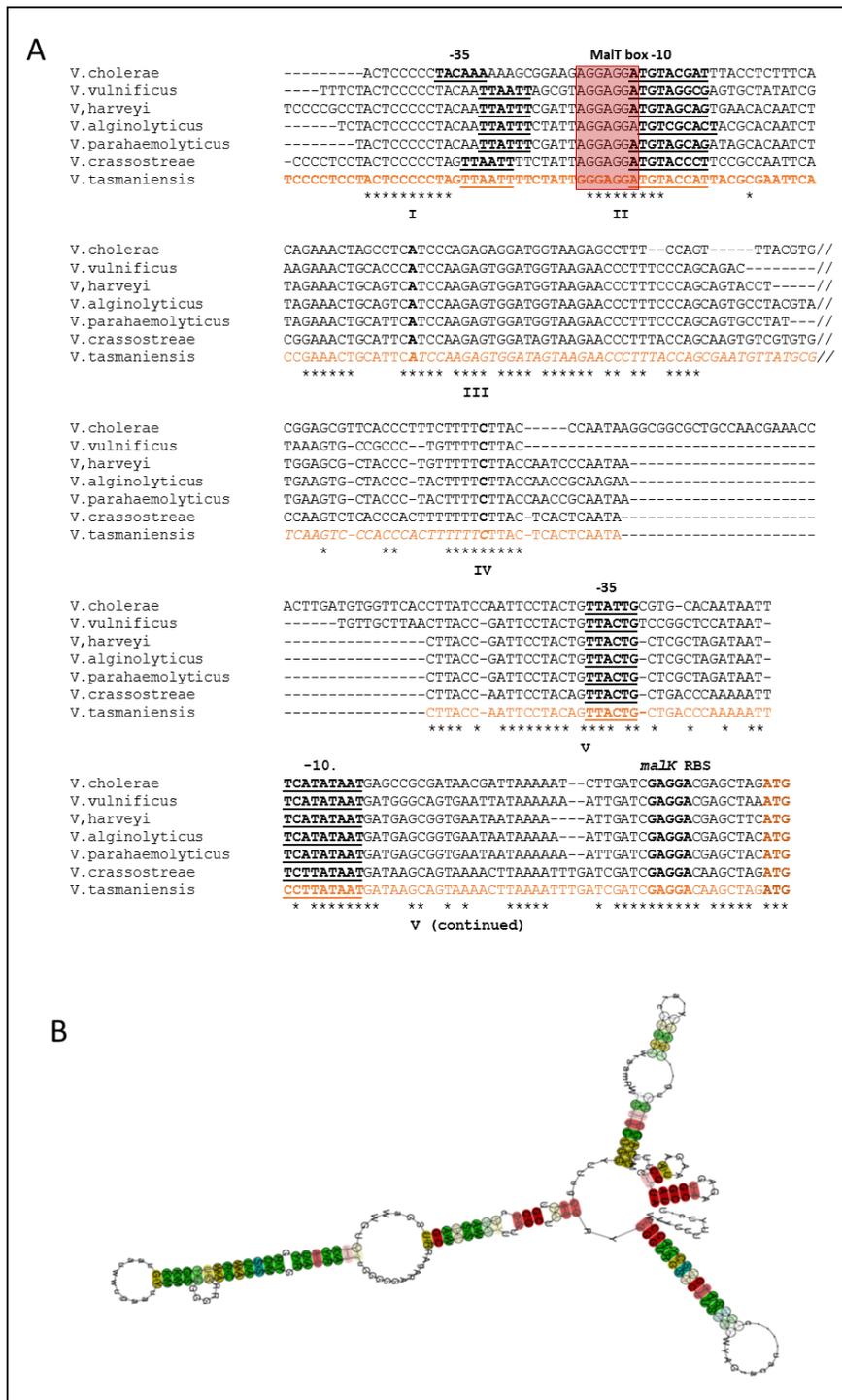


Fig. 1. A-Vsr217 is conserved amongst vibrios. The upstream regions of the *malk* gene of several vibrio reference strains, belonging to different clades (Sawabe et al, 2013b) were aligned using Muscle at the EBI (<https://www.ebi.ac.uk/Tools/msa/muscle/>). For reason of space, a non-conserved region is not shown, as indicated by the two slashes. Conserved nucleotides are indicated by (\*) under the alignment. *V. tasmaniensis* sequence is in red with *vsr217* sequence being italicized. Important features are in bold, including Vsr217 5' end (A) and 3' end (C). -35 and -10 regions of predicted promoters using BPROM (see text for details) are underlined. *malk* initiation codon is indicated in bold and red. B- Consensus secondary structure of Vsr217 was determined

by RNAalifold.

sRNAs are often more conserved at the level of secondary structure than at this of primary sequence. To look for such conservation, we used the program RNAalifold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAalifold.cgi>)(Bernhart et al, 2008) which determines the secondary structure of a sRNA on the basis of its conservation. Fig. 1B shows the consensus structure of Vsr217 based on the predicted structures in the 7 vibrios used for the alignment, underscoring structure conservation despite the low primary sequence conservation.

### **Vsr217 is induced by maltose in a MalT dependent manner and is generated from the 5' UTR of *malK*.**

The conserved position of *vsr217* upstream of *malK* and the presence of a MalT box overlapping with the putative promoter of *vsr217* (P1, Fig. 1), suggested that Vsr217 is a member of the maltose regulon and is controlled by MalT. To check this hypothesis, we tested by northern blots the production of Vsr217 and *malK* mRNA in presence or in absence of maltose in the WT strain as well as in a *malT* mutant (Fig 2). In addition, we constructed a *vsr217* mutant corresponding to a deletion encompassing the region delimited by the 5' end and 3'end of Vsr217 as determined by 5' and 3' RACE (see below and Fig 2B).

Both Vsr217 and *malK* mRNA were strongly induced in presence of maltose, whereas no expression could be detected in the presence of glucose. The corresponding bands disappeared in the *malT* mutant, indicating that both gene expression is dependent on MalT. Both in the case of Vsr217 and *malK*, we could detect, in conditions of high expression, a higher molecular weight band that could correspond to a larger transcript encompassing the two genes, whereas for *malK*, the smaller band corresponded to *malK* alone, since it was not recognized by the Vsr217 probe. Consistent with this hypothesis, in the  $\Delta vsr217$  mutant, we observed for *malK* one single band of intermediate size between the longer transcript and the *malK* transcript seen in the WT strain. This observation is compatible with this band corresponding to a transcript coming from the upstream putative promoter, shortened by the absence of *vsr217* (Fig. 1A).

We also observed in the mutant a decrease of the total amount of *malK* expressing transcripts (Fig. 2A).

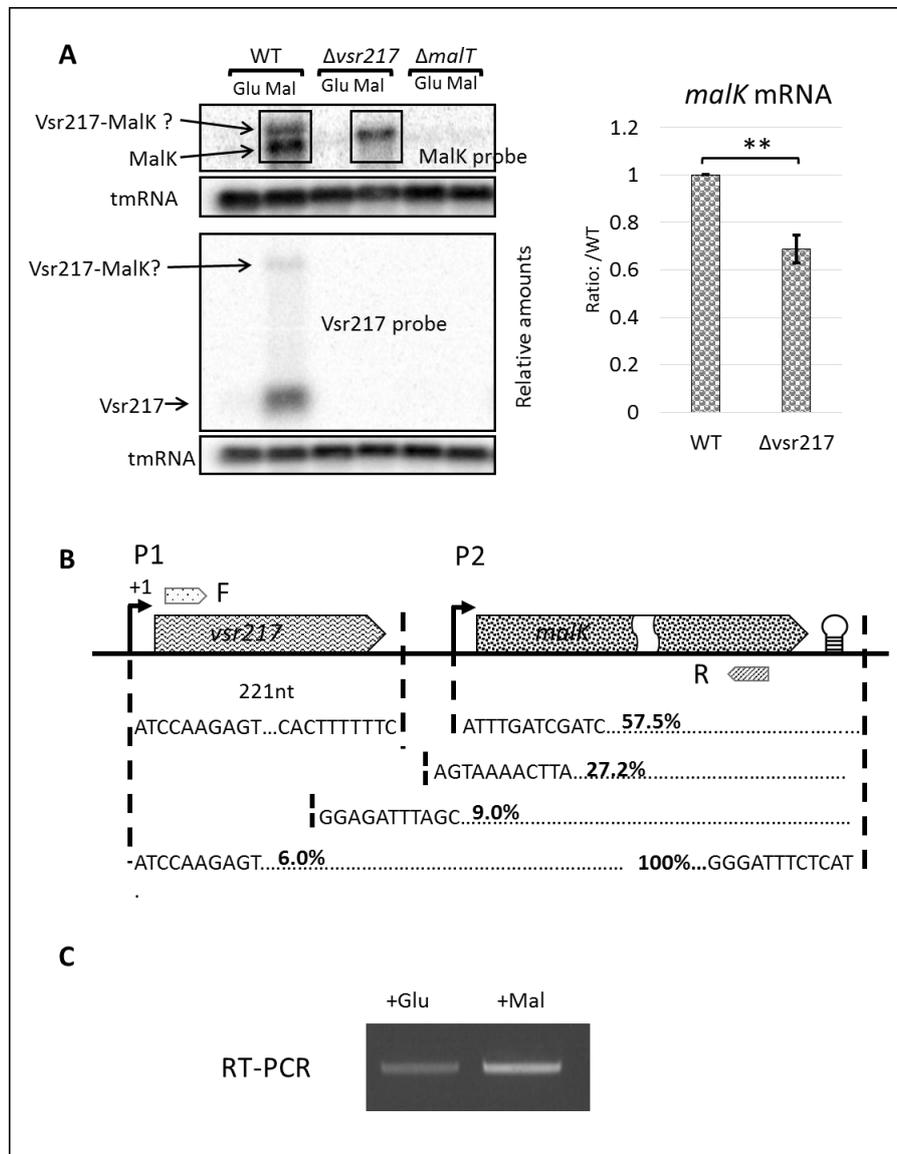


Fig. 2. Vsr217 is induced by maltose in a MalT dependent manner and is generated from the 5' UTR of *malK*. Cells were grown in Zobell + glucose or maltose since the *malT* mutant does not grow in MMS + maltose. A- Left. Upper panel: Northern blots of *malK* in MMS supplemented by glucose or maltose, in LGP32 WT,  $\Delta vsr217$ , and  $\Delta malT$ . tmRNA was used for normalisation. Lower panel: same using a probe complementary to Vsr217. Right: Quantification of the *malK* message by northern blotting. The results are from three independent northern blots and were analyzed by one-way ANOVA followed by a paired-wise t-test (\*\* p < 0.01). B- Determination of the 5' and 3' ends of Vsr217 and *malK* mRNA when the cells are grown in maltose. In each case, 20 clones were analyzed. The genetic organization is represented on top (not drawn to scale). Approximative position of the primers used for RT-PCR is indicated (F and R). C- Results of RT-PCR using LGP32 WT total RNA as template and F and R as forward and reverse primer respectively.

To further analyze the transcription pattern of the *vsr217-malK* locus, we determined by 5' RACE and 3'RACE the 5' and 3' ends of the transcripts encompassing *vsr217* and *malK*. In the case of Vsr217, all the analyzed clones displayed the same 5' and 3' end. The 5' end was compatible with a transcription stemming from the P1 promoter, and the 3' end was at the level of the highly conserved TTTTC motif. Results were very different for the *malK* transcripts. Whereas the 3' end was constant, corresponding to the end of a rho independent terminator, we identified a series of possible 5' ends. The majority (57.5%) corresponded to a possible transcription start site from the P2 promoter, whereas others were upstream of this promoter or within *vsr217* itself. 6% of the *malK* transcript had a 5' end corresponding to this of Vsr217. These results suggested strongly that *malK* could be transcribed at least in part together with *vsr217*, which is then generated from *malK* 5' UTR. A significant number of *malK* transcripts results from a processing of the 5' end of this larger transcript. To confirm the existence of such a *vsr217-malK* transcript, we performed a RT-PCR using a forward primer in *vsr217* and a reverse primer at the end of *malK*. As shown in Fig. 2C, a band could be obtained whose amount increased in presence of maltose.

### **Absence of *vsr217* leads to a growth defect in presence of maltose as a sole carbon source, due to a reduction of MalK**

Because of the *malK* mRNA level reduction seen in the  $\Delta vsr217$  mutant, we wondered whether this reduction could have a phenotypic impact on growth in presence of maltose as a sole carbon source. As seen in Fig 3A, no difference was observed between WT and mutant when grown in MMS supplemented with glucose. The WT strain grew as well in maltose as in glucose. In contrast, the *vsr217* has a slower growth rate when grown in MMS supplemented by maltose, compared to the WT. This maltose specific growth defect was corrected by the presence of a plasmid expressing *malK* under the P2 promoter, but not by a plasmid expressing Vsr217 under the P1 promoter (Fig 3B), indicating that the growth defect in the mutant is most probably due to a cis effect of the deletion of *vsr217* on *malK* expression.

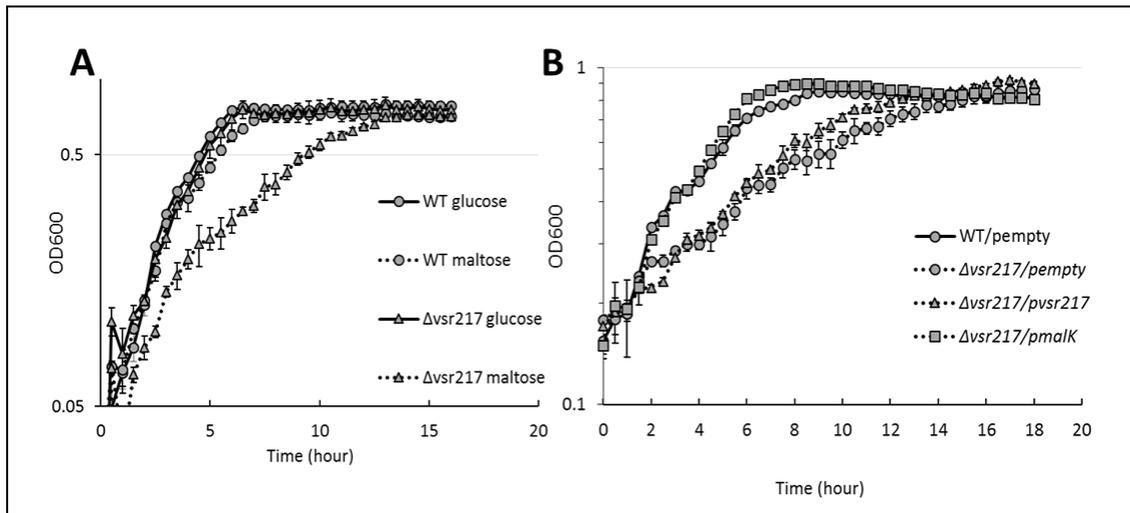


Fig. 3.  $\Delta vsr217$  has a growth defect in MMS+ maltose which is suppressed by MalK. A- Growth of LGP32 WT and  $\Delta vsr217$  strains in MMS + glucose/maltose. B- Growth of WT and  $\Delta vsr217$  strains in MMS + glucose/maltose in presence of plasmids expressing either Vsr217 (pVsr217= pGEB75, table 1) or MalK (pMalK=pGEB74, table 1). The empty plasmid pGEB12 was used as a control.

### Deletion of Vsr217 leads to a decrease of *malK* mRNA stability.

We wanted to explore further the way Vsr217 could affect *malK* expression. One possible hypothesis was an effect on mRNA stability. To check this, we determined the half-life of the *malK* and the *vsr217-malK* transcripts in the WT and  $\Delta vsr217$  strains, as well as this of Vsr217 in the mutant, by measuring by the northern blots the amounts of remaining transcripts after adding rifampicin to block transcription (Fig. 4A and 4B). We found that Vsr217 is a stable sRNA, with a half-life of approximately 7.5 min whereas *malK* and *vsr217-malK* transcripts had a half-life of less than 30s. Deletion of *vsr217* resulted in an even more unstable *malK* transcript, which decreased 90% within 30s (Fig. 4B).

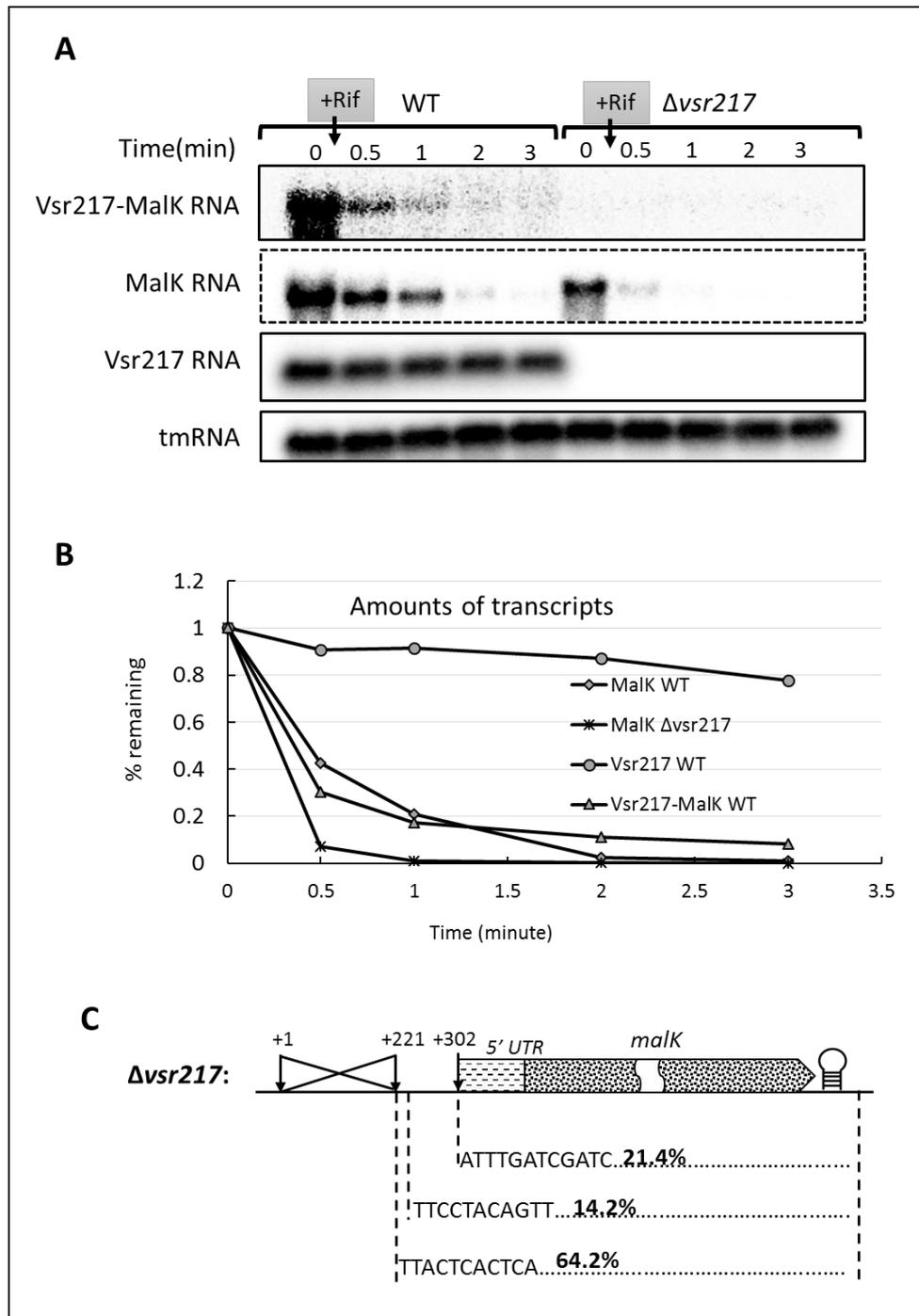


Fig. 4. Deleting *vsr217* leads to a strong instability of the *malK* message. A- Amounts of MalK and Vsr217 transcripts from cells growing in MMS + maltose ( $OD_{600} \approx 0.4$ ) were determined by northern blots over time after adding rifampicin to block transcription. B- Kinetics of degradation of *malK* and Vsr217 transcripts determined from the experiment presented in A. C- Determination of the 5' ends of the *malK* messages in the  $\Delta$ *vsr217* mutant.

We determined by 5' RACE the 5' end of the *malK* mRNA in the  $\Delta$ *vsr217* mutant. In this

strain, contrary to the WT, the majority of the transcripts (64.2%) had a 5' end corresponding to the end of the *vsr217* deletion, consistent with a transcript generated from the P1 promoter, whereas only 21.4 % of the analyzed clones corresponded to transcripts generated from the P2 promoter (Fig. 4C).

### **Regulation of the *vsr217-malK* operon by glucose and maltose.**

To further explore the regulation of *malK*, we constructed a series of *malK::gfpUV* translational fusions, where the first three codons of *malK* were fused to *gfpUV* devoid of its initiation codon. Various parts of the upstream region of *malK* were included in the fusions: a WT fragment starting from -80 nt upstream of *Vsr217* TSS (+1), an identical fragment from which *vsr217* has been deleted, thus including P1 and P2 but not *vsr217*, a fragment containing only the P1 promoter and a fragment starting from the 3' end of *vsr217* and thus containing only P2 (Fig. 5A and Fig. S1). The corresponding plasmids were introduced into *E. coli* WT strain MG1655 and Gfp fluorescence was measured during growth in M63 medium supplemented by glucose, maltose, or maltose + glucose. The latter condition was used in order to determine whether the expression of *vsr217-malK* was subjected to catabolite repression. CRP (CAMP Receptor Protein), also known as CAP (Catabolite Activator Protein) is a transcriptional activator of genes involved in the catabolism of less-preferred carbon sources, such as lactose or maltose, that is activated upon binding of cAMP. In presence of glucose, cAMP decreases, CRP is inactive and catabolite-repressed genes are not expressed (for a review, see (Bruckner & Titgemeyer, 2002). Results are presented in Fig. 5B.

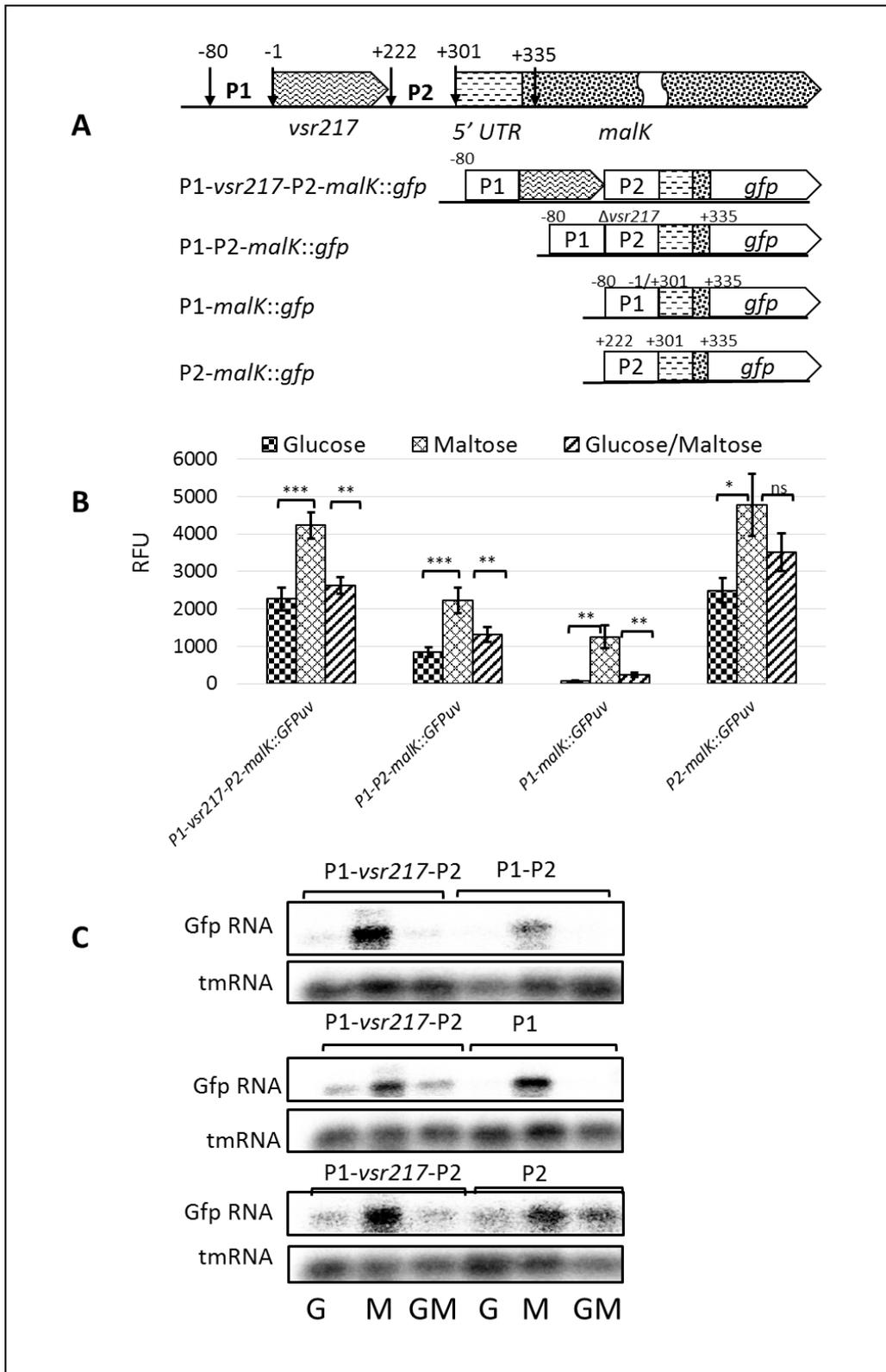


Fig. 5. Analysis of the regulatory region of *malK*. A- Plasmids expressing translational fusions between *gfpuv* deleted of its initiation codon and the first three codons of *malK* plus various upstream sequences (Fig. S1) as indicated were constructed and introduced in *E. coli* MG1655Z1 (Table 1). B- Relative fluorescence (RFU) expressed by cells containing the various fusions and

grown in M63 + glucose or maltose, or a mixture of both, as indicated, was determined as described in Materials and Methods. Results are the mean of three replicated experiments. Results of a one-way ANOVA followed by a paired-wise t-test are indicated. \*\*\*  $p < 0.005$ , \*\*  $p < 0.01$ , \*  $p < 0.05$ . C-Northern blots of the *malK-gfpuv* messages from the same strains as in B.

In the WT situation, expression of *malK* was already significant in glucose, but further induced two-fold in maltose. Adding glucose to maltose reduced by two-fold the expression, indicating that indeed *malK* expression is subjected to catabolite repression. Deleting *vsr217*, as expected, reduced *malK* expression in all three growth conditions. In the presence of P1 alone, expression of *malK* was reduced 3.5 fold, and the expression in presence of glucose was virtually null. In contrast, in the presence of P2 alone, the expression in glucose was significant and increased two-fold in maltose. Adding glucose to maltose did not significantly reduce expression (Fig. 5B).

To monitor which part of the regulation was at the transcriptional level, we also determined by northern blots the amounts of the corresponding *malK::gfp* transcripts in the different conditions and strains, using a *gfp* probe (Fig. 5C). Results were consistent with those obtained with the translational fusions, indicating that most of this regulation is at the transcriptional level. In addition, in the case of the deletion of the *vsr217* gene, the decrease of *malK::gfp* transcript can be attributed to the instability of the mRNA as shown in Fig. 4B.

Overall, these experiments indicate that the expression of *malK* is driven from two promoters. P1 is the least active but is the most contributing to regulation. It is both inducible by maltose and subjected to catabolite repression. P2 seems to be stronger and more constitutive. It is not subjected to catabolite repression but is still sensitive to maltose induction despite the absence of a predicted MalT binding site.

### **Role of the U-rich terminal motif of Vsr217.**

Vsr217 contains a conserved U-rich motif at its 3' end. We thought that this motif could play a role in generating Vsr217, either by promoting termination transcription, or by being a processing site. It could also contribute to stability of the sRNA. To test this, we constructed a

mutant deleted of the 13 last nt of *vsr217* ( $\Delta polyT$ ), and checked by northern blots the amounts of Vsr217 and of *malK* transcript in this mutant (Fig 6A). Compared to the WT strain, the  $\Delta polyT$  mutant showed a very strong reduction of Vsr217, around 100 X. In contrast, the *malK* message did not decrease, contrary to what was seen in the  $\Delta vsr217$  mutant. Neither did we observe an increase of the long *vsr217-malK* transcript as could have been expected in case of the deleted terminal motif being involved in a processing event generating Vsr217 and *malK* message. Rather, we hypothesize that this motif is involved in the stability of Vsr217.

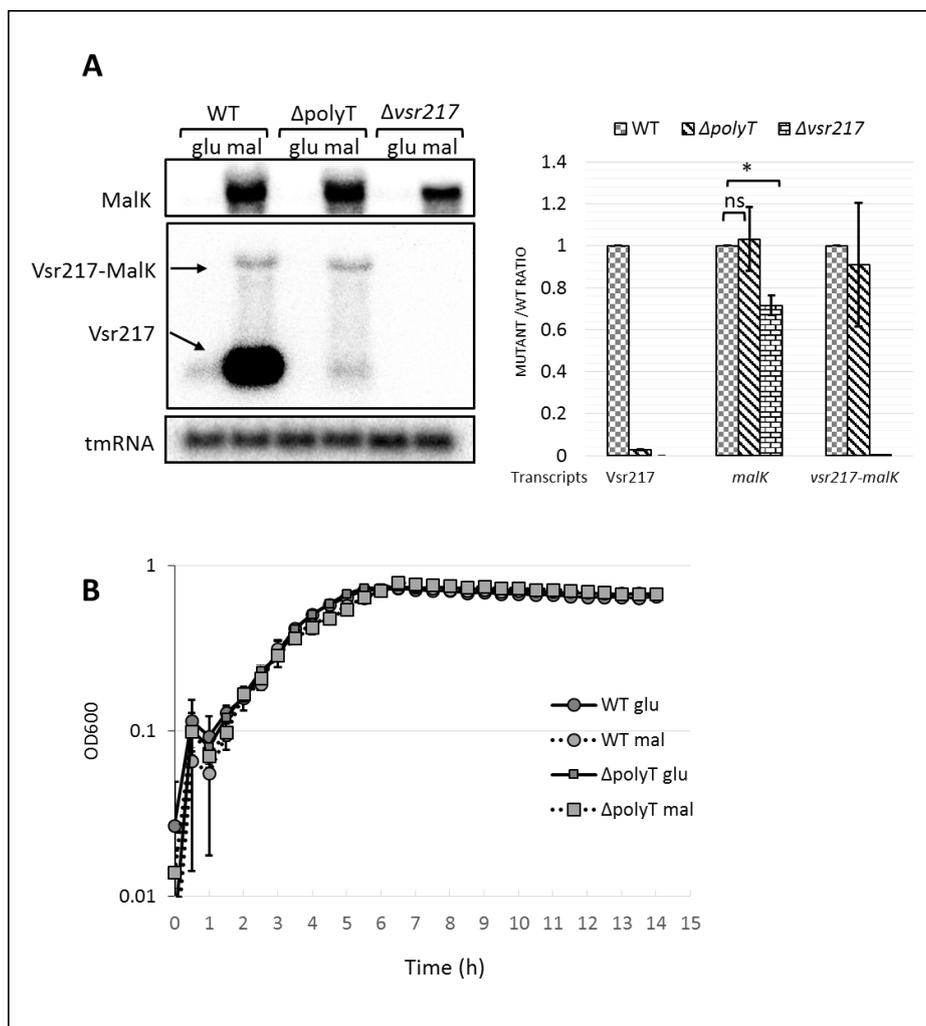


Fig. 6. Role of the polyT motif at the 3' end of *vsr217* in the expression of *vsr217* and *malK*. A- Left: Northern blots of *malK* (top) and *vsr217* (bottom) message in WT,  $\Delta polyT$  and  $\Delta vsr217$  mutant strains, grown in presence of glucose or of maltose. Right: Quantification of the northern blots. Results are the mean of two experiments. Error bars correspond to the SEM. Significant difference is indicated by a \* ( $p < 0.05$ ). B- Growth of the WT and  $\Delta polyT$  in MMS + glucose or maltose.

Since the  $\Delta polyT$  mutant did not show a decrease of the *malK* message, we examine whether it had an effect on growth in minimal medium + maltose. As expected, no difference was observed in the mutant between growth in glucose and growth in maltose (Fig. 6B). Since this mutant makes hardly any Vsr217, it confirms that the complete deletion effect on *malK* expression is due to a cis effect that does not exist in the  $\Delta polyT$  mutant, and not to the absence of Vsr217 itself.

### **Vsr217 controls the expression of the neoglucogenic enzyme fructose-bi-phosphatase.**

Overall, our results suggested that the true, physiological role of Vsr217 is not related to the utilization of maltose, but that it is used to regulate other targets in response to the presence of maltose in the medium. To identify such targets, we looked by a proteomic approach for proteins whose expression level varies between the WT and the mutant, especially when grown in maltose. To do that, whole proteins were purified by gel electrophoresis without fractionation. After in gel enzymatic digestion by nLC-ESI-MS-MS (Triple TOF, see Materials and Methods for details), resulting peptides were analyzed and quantified in each sample (three replicates for each strain/conditions) Altogether, 2104 proteins were identified in the procedure (out of 4371 CDS coded by *V. tasmaniensis* genome). In glucose, no proteins were identified whose expression changed significantly ( $|\log_2 \text{fold change}| > 1$ ,  $-\log_{10} \text{p-value} > 1.3$ ) between mutant and WT strain. In maltose, 22 proteins filled the conditions. 11 were up regulated and 11 were down regulated in the mutant (Table 3). Amongst the proteins found to decrease was MalK, confirming the effect of deleting *vsr217* on this protein level. Interestingly, amongst the 11 proteins found to decrease in the mutant, three enzymes were involved in the glycolytic/neoglucogenesis pathway: FBP (fructose-1,6-biphosphatase), GapC (glyceraldehyde-3-phosphate dehydrogenase) and PpsA (phosphoenolpyruvate synthase).

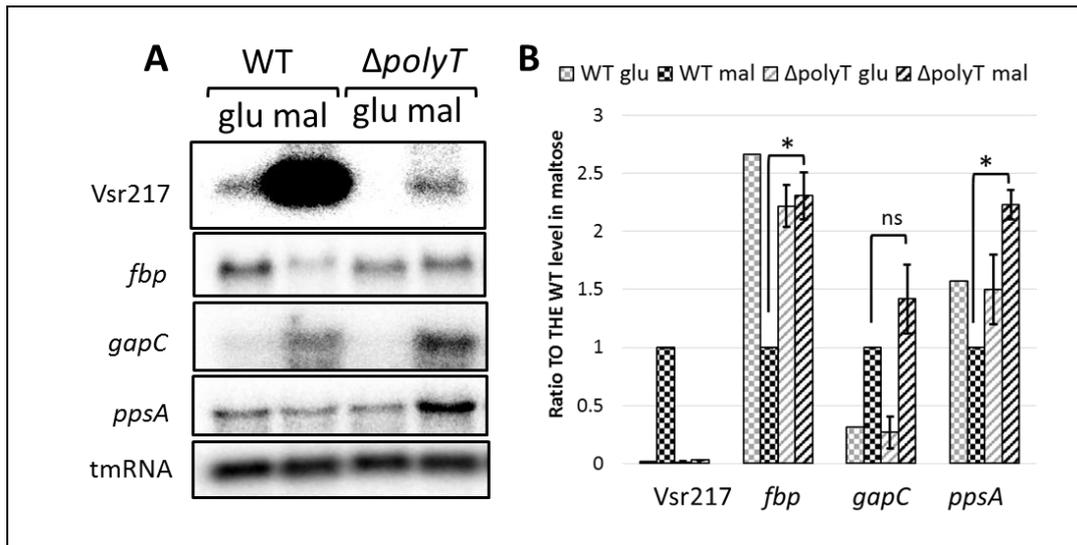


Fig. 7. Decrease of Vsr217 results in an increase of *fbp* and *ppsA* messages. A-Northern blots of 3 genes involved in neoglucogenesis/glycolysis, *fbp*, *gapC*, and *ppsA*, in the WT strain and the  $\Delta polyT$  mutant. B-Quantification of the northern blots. Results are the mean of two independent experiments. Northern blots, quantification and statistical analysis were carried out as described in previous figures.

The decrease of these proteins in the mutant could be due to an indirect effect of the decrease of MalK. To answer this question, we looked by northern blots at the level of *fbp*, *gapC* and *ppsA* mRNAs in the  $\Delta polyT$  mutant, which expresses *malK* at the same level as the WT (Fig. 7). Interestingly, each gene had a specific pattern of expression. *fbp* was expressed preferentially in glucose and repressed in maltose. This repression was abolished in the mutant, suggesting that it is mediated by Vsr217. In contrast, *gapC* mRNA was more expressed in maltose than in glucose and no significant difference in mRNA level was observed between WT and mutant. Finally, *ppsA* mRNA increased in the mutant only in presence of maltose.

We wanted to know if any of these genes could be direct target of Vsr217. To look for such direct targets of Vsr217, we did a bioinformatic search using CopraRNA, which is based on the search for conserved targets of conserved sRNAs (Wright et al, 2014). We examined in the list of the 100 best genes (ranked by false detection rate or fdr) the presence of the 22 genes encoding these proteins differentially expressed. We found only three candidates present on both lists: *fbp*, *gapC* and *malK*.

***fbp* but not *gapC* is a direct target of Vsr217**

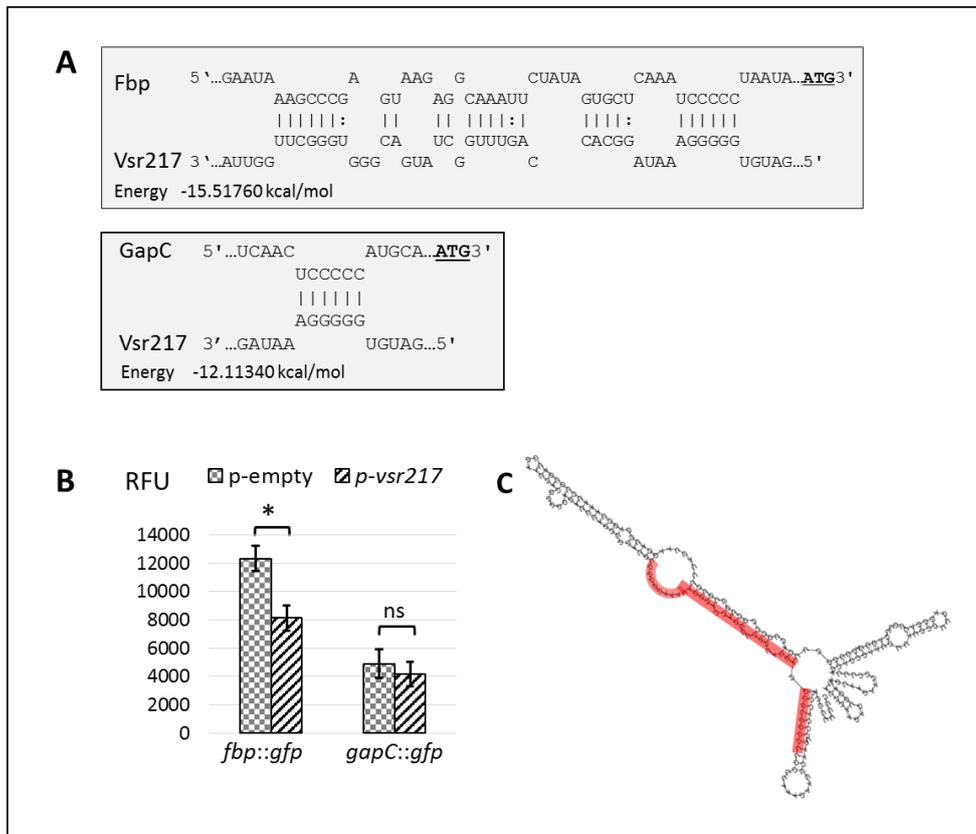


Fig. 8. Direct interaction between Vsr217 and *fbp* message. A-Interaction predicted between Vsr217 and *fbp* mRNA (top) or *gapC* mRNA (bottom) by IntaRNA (rna.informatik.uni-freiburg.de/IntaRNA/). B- Effect of pvsr217 (pGEB97, table 1) on the expression of a translational fusion including the putative interaction region between *fbp::gfp* or *gapC::gfp* as indicated. C-In red, the proposed interacting region with *fbp* mRNA according to IntaRNA is overlaid on Vsr217 secondary structure: A single-stranded conserved region A/G rich, as shown in the consensus secondary structure (Fig. 1B), is proposed to serve as a seed.

We wanted to verify if *fbp* and *gapC* were direct targets of Vsr217. To do that, we used an approach developed by Urban and Vogel (Urban & Vogel, 2007; Urban & Vogel, 2009) where the interaction is reconstituted in *E. coli*, using *gfp* as a reporter gene. We constructed two low copy plasmids where the putative interaction region of the two genes, as predicted by IntaRNA (Wright et al, 2014), including their initiation codons ATG (Fig. 8A) was fused in frame with the *gfp* gene under the control of the P<sub>Ltet</sub> promoter. The resulting plasmids, pGEB106 and pGEB107 (Table 1), were introduced in *E. coli* DH5 $\alpha$ , where, in absence of the gene coding

for the TetR repressor, the fusion genes would be expressed constitutively. A plasmid expressing Vsr217 under a  $P_{LacO}$  promoter (pGEB97) was then introduced in the strain, or as a control, the same plasmid without *vsr217* (p-empty) and the level of fluorescence was determined in the strains (Fig. 8B). In the case of *fbp*, the presence of Vsr217 led to a statistically significant reduction of *gfp* expression, whereas no difference was observed in case of *gapC*. We concluded that *fbp* mRNA but not *gapC* mRNA is a direct target of Vsr217.

## Discussion

Vsr217 is a sRNA identified in *V. tasmaniensis* LGP32 in a genome-wide search for sRNAs (Toffano-Nioche et al, 2012), which was further found to be highly induced during oyster infection (Vanhove et al, 2016). In this study, Vsr217 was found to be conserved in *Vibrio* (Fig. 1A) where it is located upstream of *malK* and downstream of a promoter possessing a MalT binding site. These features led to the hypothesis that Vsr217 was a member of the MalT regulon and was expressed in presence of maltose. This was confirmed by northern blots of Vsr217 in presence or in absence of maltose, in the WT and *malT* strains. Induction of Vsr217 by maltose could account for its high expression in oysters, which are rich in glycogen, a precursor of maltose, and is consistent with the general induction of the maltose regulon in *V. tasmaniensis* seen when in oysters (Vanhove et al, 2016).

The absence of a predicted rho independent terminator between *vsr217* and *malK* suggested that *vsr217* could be transcribed together with *malK* and that the two small transcripts could result from a processing of a large transcript. Indeed, the existence of such transcript has been evidenced both by northern blots and by Reverse-Transcription PCR (Fig. 2). However, we cannot exclude that Vsr217, which is much more stable than the *malK* transcript(s) (Fig. 4) is generated by termination of transcription at the unique 3' end that was determined by 3' RACE (Fig. 2B). The situation is more complexed in the case of *malK*: it can be expressed from 2 promoters, P1 upstream of *vsr217* and P2 in the region separating *vsr217* and *malK*. 5' end determination indicated P2 promoter as representing a little more than half of the transcripts.

The other half was distributed between transcripts with several 5' ends that could correspond to the results of processing of transcripts stemming from the P1 promoter (Fig. 2B). In Fig. 5C, we can compare the level of transcripts stemming from the two promoters examined individually, using northern blots of Gfp fusions. These two promoters generated similar levels of transcripts both when compared to each other, and when compared to the WT situation when the two promoters are present (Fig. 5C). In addition, in absence of *vsr217*, a situation where P1 and P2 promoters are close to each other, we can see a strong reduction of *gfp* transcripts. This effect on *malK* expression is also visible at the translational level (Fig. 5B).

There are several ways, not exclusive, to interpret these results, in term of effect of the absence of *vsr217*. One effect could be at the level of message stability. Indeed, as also visible in Fig. 4A, in the  $\Delta vsr217$ , *malK* message becomes much more instable. Another effect could be a competition between the two promoters: in absence of a strong terminator after *vsr217*, transcripts coming from P1 could interfere with transcription at P2. Finally, the results obtained with the Gfp translational fusion, compared to the level of the fusion transcript (see Fig. 5b and 5.C) suggest that transcripts coming from P1 are less efficiently translated than transcripts coming from P2. In the  $\Delta vsr217$  mutant, 5' end determination has shown these P1 originating transcripts are the majority.

Deleting the 13 last nt corresponding to a conserved region at the 3' end of *vsr217* led to a nearly complete absence of *vsr217*. The easiest way to interpret this result is to hypothesize that this 3' end is important for Vsr217 stability, and that, in its absence, the sRNA becomes degraded. Surprisingly, in this mutant, there was no reduction of *malK* expression, confirming that a cis effect of deleting *vsr217* and not its absence, was responsible for MalK decrease.

Given the stability of Vsr217, its regulation, the absence of phenotype in term of maltose utilization, we postulated that Vsr217 had a function as a *trans*-acting sRNA. A proteomic study comparing mutant and WT growing in Zobell + glucose/maltose, highlighted 11 proteins that increased in the mutant, and could be candidates for genes being negatively regulated by Vsr217. Interestingly three of these genes, *fbp*, coding for fructose-1,6-biphosphatase, *gapC*,

one of three genes in *V. tasmaniensis* which codes for glyceraldehyde-3-phosphate dehydrogenase and *ppsA*, coding for a phosphoenolpyruvate synthase, were genes coding for enzymes of the glycolytic/neoglucogenesis pathways. Two of these, *fbp* and *ppsA*, had increased mRNA level in the  $\Delta polyT$  mutant in maltose compared to the WT in the same condition. We used CopraRNA, a bioinformatic method that computes possible target genes of a given sRNA, and examined the list of the 100 best candidates. We found in this list two of these genes, *fbp* and *gapC*. Out of these two, we could demonstrate a direct interaction between Vsr217 and *fbp* mRNA using a *fbp::gfp* translational fusion in *E. coli* (Fig. 8B).

In summary, as schematically drawn in Fig. 9, Vsr217 increases in presence of maltose as a sole carbon source, and inhibits expression of at least two enzymes of the glycolytic pathway, Fbp, which is a direct target and PpsA, most likely indirectly. In the case of GapC, it is still possible that Vsr217 decrease its expression indirectly at the translational level, since we did not find a significant effect on the transcript level. Interestingly, whereas most steps of glycolysis are reversible, two steps are not and don't use the same enzymes for glycolysis as for neoglucogenesis: both Fbp and PpsA work only in the direction of neoglucogenesis. Hence, in presence of maltose, where glucose 6-phosphate is made from, the decrease of these two enzymes due to the high expression of Vsr217 stimulate glycolysis rather than neoglucogenesis.

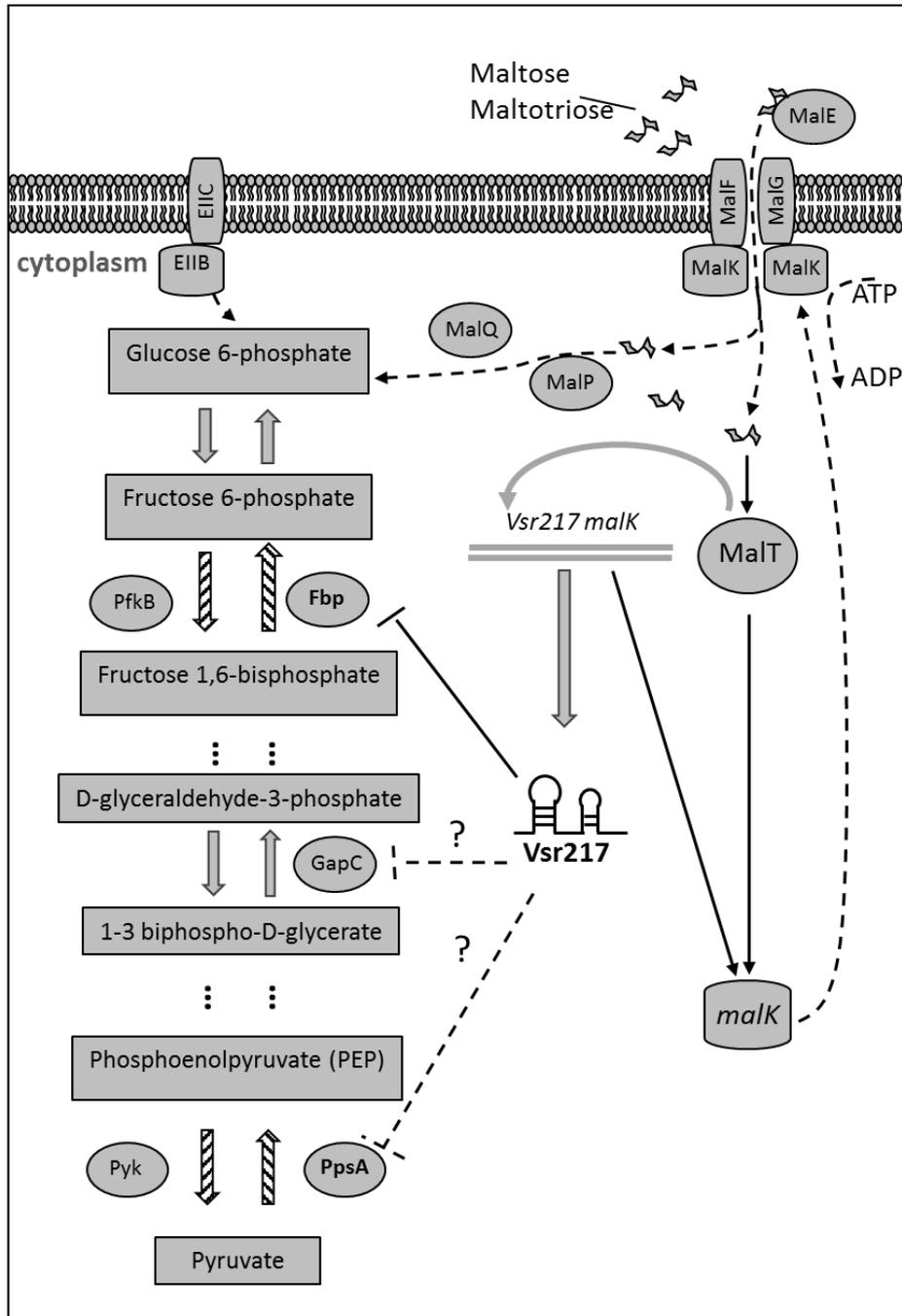


Fig. 9. Diagrammatic representation of the role of Vsr217 in the metabolism of *V. tasmaniensis*. In the presence of maltose or maltotriose as a carbon source, the sugars activate MalT, turning on MalT regulated genes, including maltose transport genes such as *malK*, *malE*, *malF,G*, and maltose metabolic genes *malPQ*. Vsr217 is then highly expressed as a result of the activation of MalT and represses, by direct interaction the expression of *fbp*, and indirectly, expression of *ppsA*, inhibiting neoglucogenesis and stimulating glycolysis. Only the relevant steps of glycolysis are indicated.

## Materials and methods

### Bacterial strains and plasmid

Bacterial strains and plasmids used in this study are listed in Table 1. *V. tasmaniensis* LGP32 mutants were constructed by allele replacement through homologous recombination as described previously (Nguyen et al, 2018a). The presence of the desired mutations was confirmed by sequencing. Plasmids were constructed by one-step isothermal assembly (Gibson assembly) (Gibson, 2011; Yanofsky, 2000). Primers used for DNA amplification are listed in Table 2.

### Media and bacterial growth

*E. coli* strains were cultured at 37 °C in Luria–Bertani (LB) broth or M63 medium (3 g/L  $\text{KH}_2\text{PO}_4$ , 7 g/L  $\text{K}_2\text{HPO}_4$ , 2 g/L  $(\text{NH}_4)_2\text{SO}_4$ ,  $0.5 \times 10^{-3}$  g/L  $\text{FeSO}_4$ ,  $2 \times 10^{-3}$  M  $\text{MgSO}_4$ , 10 g/L NaCl) supplemented with 2 g/L D-glucose or D-maltose. *V. tasmaniensis* LGP32 and its derivative were grown at 20 °C in Zobell medium (4 g/L peptone, 1 g/L yeast extract, 0.1 g/L ferric phosphate, 30 g/L sea salt) or minimal medium with salt (MMS=M63 with 30 g/L NaCl) supplemented with 2 g/L D-glucose or D-maltose, as indicated. Antibiotics were used as follow: chloramphenicol (Cm) at 20 µg/ml and ampicillin (Amp) at 100 µg/ml for *E. coli* and Cm at 2 µg/ml for LGP32. IPTG was used at 0.1 mM.

To measure growth of LGP32, bacteria (3 replicate cultures in each case) were cultured overnight in MMS supplemented with 2 g/L D-glucose or D-maltose. If needed, Cm was added. Overnight cultures were diluted to  $\text{OD}_{600\text{nm}}$  ( $\text{OD}_{600}$ )  $\approx 0.05$  in the same medium and growth was monitored in a 96 well plate, by measuring cell density at  $\text{OD}_{600}$  at regular time intervals in a CLARIOstar (BMG Labtech, France) microplate reader.

### RT-PCR and northern blotting

Overnight cultured bacteria were diluted to  $\text{OD}_{600} \approx 0.05$  in the indicated medium and grown with continuous shaking at 180 rpm at 20 °C for LGP32 or 37 °C for *E. coli*. When  $\text{OD}_{600}$  reached  $\approx 0.4$ , bacteria were collected by centrifugation and resuspended in TRI Reagent™ (Sigma) before total RNA extraction and purification, which was carried out using Direct-zol MiniPrep Plus (Zymo research). DNA was removed by TURBO™ DNase (Ambion)

treatment. RNA was further purified by phenol-chloroform extraction and RNA precipitation. 10 µg purified RNA was used for northern blotting as described previously (Toffano-Nioche et al, 2012). Oligonucleotide probes (Table 2) complementary to the gene to be assayed were labeled at their 3' end using terminal transferase (Fermentas, USA) and [ $\alpha$ -<sup>32</sup>P] dCTP, according to the manufacturer instructions. tmRNA was used as a loading control. Transcripts were quantified by image J and normalized to the signal of tmRNA.

For RT-PCR, 1 µg RNA was used as a template for the first strand synthesis catalyzed by SuperScript™ III Reverse Transcriptase (Invitrogen). Amplification of the Vsr217-MalK transcript was then carried out using DreamTaq DNA polymerase (Thermo Fisher Scientific) and primers Vsr217-RT-F and MalK-RT-R (Table 2).

### **5' and 3' rapid amplification of cDNA ends (RACE)**

Overnight cultures of LGP32 WT and  $\Delta$ *vsr217* were diluted to OD<sub>600</sub>  $\approx$  0.05 and grown in MMS supplemented with 2 g/L D-maltose. Bacteria were collected by centrifugation when OD<sub>600</sub> reached  $\approx$  0.4, and resuspended in TRI Reagent™ followed by RNA extraction, DNase treatment and RNA purification. 5' and 3' ends of Vsr217 and MalK transcripts were determined using the 5'/3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen). RNA primers used are listed in Table 2. In each case, the subsequent PCR products were cloned in the pJET1.2 vector (Thermo Fisher Scientific). 20 plasmids were prepared from randomly chosen colonies and inserts were sequenced using oligonucleotides pJET1.2-F/R (Table 2).

### **RNA stability measurement**

LGP32 WT and  $\Delta$ *vsr217* were grown overnight in MMS supplemented with 2 g/L D-maltose at 20 °C. Bacteria were diluted to OD<sub>600</sub>  $\approx$  0.05 and grown in the same medium until OD<sub>600</sub>  $\approx$  0.4 and rifampicin was added to a final concentration of 500 µg/ml. 10 ml of bacteria were collected for RNA extraction at 0, 0.5, 1.0, 2.0, and 3.0 minutes after the addition of rifampicin. Northern blot analysis was performed to determine the amount of *vsr217* and *malK* transcript.

### **Gfp fluorescence measurement**

To monitor Gfp fluorescence, strains to be assayed were diluted to OD  $\approx$  0.05 in M63 + appropriate sugar + Cm (Fig 5) or LB supplemented with Cm, Amp and IPTG (Fig 8) and

growth and fluorescence were monitored at regular time point intervals at 37°C in a CLARIOstar microplate reader, using 200 µl of culture. Two different Gfp were used in this study, Gfpuv (Exc 395/20 nm, Em 509/20 nm) for results presented in Fig. 5 and eGfp (Exc 480/20 nm/Em 520/10 nm) for results presented in Fig. 8. Interactions between Vsr217 and its targets were checked using a *gfp*-based translational fusion system as previously described (Urban & Vogel, 2007). A strain containing a plasmid with no *gfp* expression was used as a control for background fluorescence, which was deducted from the measured fluorescence units (FU). Relative fluorescence units (RFU = FU/OD600) were then calculated at the different time points. After an initial increase, relative fluorescence reached a plateau during exponential phase, starting at OD ≈ 0.2. The presented values are the mean of RFU measurements between OD ≈ 0.2 and 0.3 over three replicated experiments.

## Proteomics

*V. tasmaniensis* LGP32 and  $\Delta$ *vsr217* were grown in triplicates in Zobell with glucose or maltose until OD600 ≈ 0.4 (12 samples in total). After centrifugation of 2 ml, cells were washed in Tris/EDTA pH7.5 + 20% sucrose, and then resuspended in NuPAGE™ LDS buffer (Thermo Fisher Scientific) to a final concentration of OD600=20 equivalent. Samples were heated at 95°C for 10 min before loading 10 µl of each sample on a 4-12% polyacrylamide gradient gel (Invitrogen). After 5 min migration to let the samples enter the gels, they were stained in Coomassie blue, and then destained.

For MS/MS, proteins were subjected to an in-gel enzymatic digestion with trypsin. LC-MS/MS (Triple-TOF) was carried out (gradient of 120 mn) and the peptides were identified by Mascot and Andromeda and quantified by Maxquant. Statistical analyses were carried out using Perseus (<https://maxquant.net/perseus/>)

## References

- Bernhart, S. H., I. L. Hofacker, S. Will, A. R. Gruber and P. F. Stadler (2008). "RNAalifold: improved consensus structure prediction for RNA alignments." *BMC Bioinformatics* **9**: 474.
- Boos, W. and H. Shuman (1998). "Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation." *Microbiol Mol Biol Rev* **62**(1): 204-229.

Brantl, S. and R. Bruckner (2014). "Small regulatory RNAs from low-GC Gram-positive bacteria." RNA Biol **11**(5): 443-456.

Bruckner, R. and F. Titgemeyer (2002). "Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization." FEMS Microbiol Lett **209**(2): 141-148.

Chao, Y. and J. Vogel (2016). "A 3' UTR-Derived Small RNA Provides the Regulatory Noncoding Arm of the Inner Membrane Stress Response." Mol Cell **61**(3): 352-363.

Duperthuy, M., P. Schmitt, E. Garzon, A. Caro, R. D. Rosa, F. Le Roux, N. Lautredou-Audouy, P. Got, B. Romestand, J. de Lorgeril, S. Kieffer-Jaquinod, E. Bachere and D. Destoumieux-Garzon (2011). "Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*." Proc Natl Acad Sci U S A **108**(7): 2993-2998.

Gay, M., T. Renault, A. M. Pons and F. Le Roux (2004). "Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations." Dis Aquat Organ **62**(1-2): 65-74.

Gibson, D. G. (2011). Chapter fifteen - Enzymatic Assembly of Overlapping DNA Fragments. Methods in Enzymology. C. Voigt, Academic Press. **498**: 349-361.

Gibson, D. G. (2011). "Enzymatic assembly of overlapping DNA fragments." Methods Enzymol **498**: 349-361.

Guo, M. S., T. B. Updegrave, E. B. Gogol, S. A. Shabalina, C. A. Gross and G. Storz (2014). "MicL, a new sigmaE-dependent sRNA, combats envelope stress by repressing synthesis of Lpp, the major outer membrane lipoprotein." Genes Dev **28**(14): 1620-1634.

Johansson, J., P. Mandin, A. Renzoni, C. Chiaruttini, M. Springer and P. Cossart (2002). "An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*." Cell **110**(5): 551-561.

Kawano, M., A. A. Reynolds, J. Miranda-Rios and G. Storz (2005). "Detection of 5'- and 3'-UTR-derived small RNAs and cis-encoded antisense RNAs in *Escherichia coli*." Nucleic Acids Res **33**(3): 1040-1050.

Lakhal, F., S. Bury-Mone, Y. Nomane, N. Le Goic, C. Paillard and A. Jacq (2008). "DjIA, a membrane-anchored DnaJ-like protein, is required for cytotoxicity of clam pathogen *Vibrio tapetis* to hemocytes." Appl Environ Microbiol **74**(18): 5750-5758.

Le Roux, F., J. Binesse, D. Saulnier and D. Mazel (2007). "Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector." Appl Environ Microbiol **73**(3): 777-784.

Le Roux, F., M. Zouine, N. Chakroun, J. Binesse, D. Saulnier, C. Bouchier, N. Zidane, L. Ma, C. Rusniok, A. Lajus, C. Buchrieser, C. Medigue, M. F. Polz and D. Mazel (2009). "Genome sequence of *Vibrio splendidus*: an abundant planktonic marine species with a large genotypic diversity." Environ Microbiol **11**(8): 1959-1970.

Liu, J. M. and A. Camilli (2010). "A broadening world of bacterial small RNAs." Curr Opin Microbiol **13**(1): 18-23.

Loh, E., O. Dussurget, J. Gripenland, K. Vaitkevicius, T. Tiensuu, P. Mandin, F. Repoila, C. Buchrieser, P. Cossart and J. Johansson (2009). "A trans-acting riboswitch controls expression of the virulence regulator PrfA in *Listeria monocytogenes*." Cell **139**(4): 770-779.

Manna, A. C., S. Kim, L. Cengher, A. Corvaglia, S. Leo, P. Francois and A. L. Cheung (2018). "small RNA Teg49 is derived from a *sarA* transcript and regulates virulence genes independent of *sarA* in *Staphylococcus aureus*." Infect Immun **86**(2).

Melior, H., S. Li, R. Madhugiri, M. Stotzel, S. Azarderakhsh, S. Barth-Weber, K. Baumgardt, J. Ziebuhr and E. Evguenieva-Hackenberg (2019). "Transcription attenuation-derived small RNA rTrpL regulates tryptophan

biosynthesis gene expression in trans." Nucleic Acids Res.

Miotto, P., F. Forti, A. Ambrosi, D. Pellin, D. F. Veiga, G. Balazsi, M. L. Gennaro, C. Di Serio, D. Ghisotti and D. M. Cirillo (2012). "Genome-wide discovery of small RNAs in *Mycobacterium tuberculosis*." PLoS One **7**(12): e51950.

Nguyen, A. N., E. Disconzi, G. M. Charriere, D. Destoumieux-Garzon, P. Bouloc, F. Le Roux and A. Jacq (2018). "csrB gene duplication drives the evolution of redundant regulatory pathways controlling expression of the major toxic secreted metalloproteases in *Vibrio tasmaniensis* LGP32." mSphere **3**(6) doi:10.1128/mSphere.00582-18

Sawabe, T., Y. Ogura, Y. Matsumura, G. Feng, A. R. Amin, S. Mino, S. Nakagawa, T. Sawabe, R. Kumar, Y. Fukui, M. Satomi, R. Matsushima, F. L. Thompson, B. Gomez-Gil, R. Christen, F. Maruyama, K. Kurokawa and T. Hayashi (2013). "Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov." Front Microbiol **4**: 414.

Schlegel, A., A. Bohm, S. J. Lee, R. Peist, K. Decker and W. Boos (2002). "Network regulation of the *Escherichia coli* maltose system." J Mol Microbiol Biotechnol **4**(3): 301-307.

Toffano-Nioche, C., A. N. Nguyen, C. Kuchly, A. Ott, D. Gautheret, P. Bouloc and A. Jacq (2012). "Transcriptomic profiling of the oyster pathogen *Vibrio splendidus* opens a window on the evolutionary dynamics of the small RNA repertoire in the *Vibrio* genus." RNA **18**(12): 2201-2219.

Urban, J. H. and J. Vogel (2007). "Translational control and target recognition by *Escherichia coli* small RNAs in vivo." Nucleic Acids Res **35**(3): 1018-1037.

Urban, J. H. and J. Vogel (2009). "A green fluorescent protein (GFP)-based plasmid system to study post-transcriptional control of gene expression in vivo." Methods Mol Biol **540**: 301-319.

Val, M. E., O. Skovgaard, M. Ducos-Galand, M. J. Bland and D. Mazel (2012). "Genome engineering in *Vibrio cholerae*: a feasible approach to address biological issues." PLoS Genet **8**(1): e1002472.

van der Meulen, S. B., A. de Jong and J. Kok (2016). "Transcriptome landscape of *Lactococcus lactis* reveals many novel RNAs including a small regulatory RNA involved in carbon uptake and metabolism." RNA Biol **13**(3): 353-366.

Vanhove, A. S., M. Duperthuy, G. M. Charriere, F. Le Roux, D. Goudenege, B. Gourbal, S. Kieffer-Jaquinod, Y. Coute, S. N. Wai and D. Destoumieux-Garzon (2015). "Outer membrane vesicles are vehicles for the delivery of *Vibrio tasmaniensis* virulence factors to oyster immune cells." Environ Microbiol **17**(4): 1152-1165.

Vanhove, A. S., T. P. Rubio, A. N. Nguyen, A. Lemire, D. Roche, J. Nicod, A. Vergnes, A. C. Poirier, E. Disconzi, E. Bachere, F. Le Roux, A. Jacq, G. M. Charriere and D. Destoumieux-Garzon (2016). "Copper homeostasis at the host vibrio interface: lessons from intracellular vibrio transcriptomics." Environ Microbiol **18**(3): 875-888.

Vidal-Ingigliardi, D., E. Richet and O. Raibaud (1991). "Two MalT binding sites in direct repeat. A structural motif involved in the activation of all the promoters of the maltose regulons in *Escherichia coli* and *Klebsiella pneumoniae*." J Mol Biol **218**(2): 323-334.

Wagner, E. G. and P. Romby (2015). "Small RNAs in bacteria and archaea: who they are, what they do, and how they do it." Adv Genet **90**: 133-208.

Wright, P. R., J. Georg, M. Mann, D. A. Sorescu, A. S. Richter, S. Lott, R. Kleinkauf, W. R. Hess and R. Backofen (2014). "CoproRNA and IntaRNA: predicting small RNA targets, networks and interaction domains." Nucleic Acids Res **42**(Web Server issue): W119-123.

Zhang, Y., D. Yan, L. Xia, X. Zhao, G. Osei-Adjei, S. Xu, X. Sheng and X. Huang (2017). "The malS-

5'UTR regulates *hisG*, a key gene in the histidine biosynthetic pathway in *Salmonella enterica* serovar Typhi." Can J Microbiol **63**(4): 287-295.

**Table 1. Strains and plasmids used in this study**

Strain or plasmid	Relevant characteristics	References
<i>V. tasmaniensis</i>		
<b>LGP32</b>	Pathogen of oyster <i>C. gigas</i>	(Le Roux et al, 2009)
<b><math>\Delta</math><i>vsr217</i></b>	LGP32 carrying a deletion of <i>vsr217</i>	This study
<b><math>\Delta</math><i>malT</i></b>	LGP32 carrying a deletion of <i>malT</i>	This study
<b><math>\Delta</math><i>polyT</i></b>	LGP32 carrying a deletion of <i>polyT</i> at the 3' end of <i>vsr217</i>	This study
<i>E. coli</i>		
<b>DH5<math>\alpha</math></b>	F <sup>-</sup> <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20</i> <i><math>\phi</math>80dlacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )U169, <i>hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ), $\lambda$ <sup>-</sup>	Lab collection
<b>MG1655</b>	F <sup>-</sup> $\lambda$ <sup>-</sup> , <i>ilvG</i> <sup>-</sup> , <i>rfb-50</i> , <i>rph-1</i> , Z1	Lab collection
<b><math>\Pi</math>3813</b>	<i>E. coli</i> K12 <i>lacIQ</i> , <i>thi1</i> , <i>supE44</i> , <i>endA1</i> , <i>recA1</i> , <i>hsdR17</i> , <i>gyrA462</i> , <i>zei298::tn10</i> [Tc], <i><math>\Delta</math>thyA::(erm-pir116)</i> [Er]; cloning host	(Le Roux et al, 2007)
<b>GEB883</b>	<i>E. coli</i> K12 <i><math>\Delta</math>dapA::erm pir</i> [Er] <i>RP4-2 <math>\Delta</math>recA gyrA462</i> , <i>zei298::Tn10</i> [Tc]; donor strain for conjugation	(Nguyen et al, 2018a)
Plasmids		
<b>Coordinates refer to Fig. 1A and Fig S1 except for pGEB106 and 107</b>		
<b>pSW7848</b>	Suicide vector with an R6K origin, requiring the Pir protein for its replication, pBAD- <i>ccdB</i> , Cm <sup>R</sup>	(Val et al, 2012; Yanofsky, 2000)
<b>pSW7848-<math>\Delta</math><i>vsr217</i></b>	pSW7848 carrying the mutant allele $\Delta$ <i>vsr217</i> , Cm <sup>R</sup>	This study
<b>pSW7848-<math>\Delta</math><i>malT</i></b>	pSW7848 carrying an in-frame deletion of <i>malK</i> , Cm <sup>R</sup>	This study
<b>pSW7848-<math>\Delta</math><i>polyT</i></b>	pSW7848 carrying the <i>polyT</i> 13 nt deletion at the 3' end of <i>vsr217</i> , Cm <sup>R</sup>	This study
<b>pGEB12</b>	Replicative, pSU18 with <i>ori</i> p15A, <i>ori</i> T <sub>RP4</sub> , Cm <sup>R</sup>	(Lakhal et al, 2008)
<b>pGEB74</b>	pGEB12, [+222, +301]- <i>malK</i> , Cm <sup>R</sup>	This study
<b>pGEB75</b>	pGEB12, [-80, -1]- <i>vsr217</i> , Cm <sup>R</sup>	This study
<b>pGEB77</b>	pGEB12, [-80, -1]- <i>vsr217</i> -[+222, +301]- <i>malKATG::gfpuv</i> , Cm <sup>R</sup>	This study
<b>pGEB78</b>	pGEB12, [-80, -1][ 222, +301]- <i>malKATG::gfpuv</i> , Cm <sup>R</sup>	This study
<b>pGEB80</b>	pGEB12, <i>gfpuv</i> without promoter, Cm <sup>R</sup> , (control plasmid to determine cellular fluorescence)	This study
<b>pGEB81</b>	pGEB12, [+222, +301]- <i>malKATG::gfpuv</i> , Cm <sup>R</sup>	This study
<b>pGEB100</b>	pGEB12, [-80, -1][ 222, +301]- <i>malKATG::gfpuv</i> , Cm <sup>R</sup>	This study
<b>pZE12MCS</b>	Based on pZE12-Luc, P <sub>LacO</sub> , <i>colE1</i> , Amp <sup>R</sup>	(Urban & Vogel, 2007)
<b>pGEB97</b>	pZE12MCS, P <sub>LacO</sub> - <i>vsr217</i> , Amp <sup>R</sup>	This study
<b>pXG-10</b>	pSC101* origin of replication (low copy), P <sub>LetO</sub> promoter, <i>egfp</i> <sup>+</sup> , Cm <sup>R</sup> ,	(Urban & Vogel, 2007)
<b>pGEB106</b>	pXG-10,[-128, +3]- <i>fbp::egfp</i> , Cm <sup>R</sup> (+1= A of the initiation codon)	This study

**pGEB107**

pXG-10, [-200, +3]-*gapC*::*egfp*, Cm<sup>R</sup> (+1= A of the initiation codon) This study

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**Table 2 oligonucleotides used in this study**

Non-target bases complementary to the cloning vector are indicated by lower case letters

Name	Sequence (5'-3')	Targets/Goal
<b>Northern blot probes</b>		
NB-vs-MalK	CATACCTACACCACGTTTT- GATGGCC	<i>malK</i>
NB-vs-Vsr217	CCCGTGATTCAATCACAGATTTAGCTCGA	<i>Vsr217</i>
NB-GFPuv	GACAGAAAATTTGTGCCCATTAACA	<i>Gfpuv</i>
NB-vs-Fbp	ATCTCACGGTTAACAATTTTTGCAG	<i>fbp</i>
NB-vs-GapC	GAATGATGGGTTGAGTCTGTTCTGCG	<i>gapC</i>
NB-vs-tmRNA	AGCCACGCTAGCTAGTTTGGGTTTGATCT	tmRNA
<b>RT-PCR</b>		
Vsr217-RT-F	ACCAGCGAATGTTATGCGTT	Vsr217
MalK-RT-R	CAAGCGACGACATGCTCTAC	<i>malK</i>
<b>5' and 3' RACE</b>		
Oligo d(T)-Anchor primer	GACCACGCG- TATCGATGTCGACTTTTTTTTTTTTTTTT	RACE product cloning
PCR Anchor primer	GACCACGCGTATCGATGTCGAC	RACE product cloning
5' Malk-GSP1	GAATACCATACCTACACCACGTTTT	<i>malK</i>
5' Malk-GSP2	ACGTAATATCTTCTAGACCTGCGAT	<i>malK</i>
5' Malk-GSP3	GAACTCACCTTCGTTGATGTCTAAG	<i>malK</i>
5' Vsr217-GSP1	GGTGGGACTTGAATTCTTTAACCAA	Vsr217
5' Vsr217-GSP2	CACCCGTCATAGCCAAACTG	Vsr217
5' Vsr217-GSP3	CCAATAAATGAACAATGCGTCAAC	Vsr217
3' Malk-GSP1	TGCGACGATTGAAGCTGATG	<i>malK</i>
3' Malk-GSP2	GGTATTCCTGCACACCGTTG	<i>malK</i>
3' Vsr217-GSP1	ACCAGCGAATGTTATGCGTT	Vsr217
3' Vsr217-GSP2	CTGCCCGCTGTTGTCTTTAT	Vsr217
<b><i>gfp</i> - translational fusion</b>		
pGEB12-F	CACTGGCCGTCGTTTTACAACGTCG	pGEB12
pGEB12-R	CAAGCTTGCATGCCTGCAGGTCGAC	pGEB12
GFPuv-F	aagctagatggcgagtAGTAAAGGAGAA- GAACTTTTC	<i>gfpuv</i>
GFPuv-R	ctaaaaagacgaaccgaTTATTT- GTAGAGCTCATCCATG	<i>gfpuv</i>
pGEB77-F	aggeatgcaagctt- gCCCTTTATCTACTCCTCCCCTCC- TACTCCCCCTAG	[-80, +335]
pGEB77-R	ttcttctccttactACTCGCCATCTAGCTT- GTCCTCGATCGATCAAATT	[-80, +335]
pGEB78-F	TTACTCACTCAATACTTACCAATTC	Removing [+1, +221] from

pGEB78-R	gtattgagtgagtaaGAATGCAG- TTTCGGTGAATTCGCGTAATGGTACAT	pGEB77 Removing [+1, +221] from pGEB77
pGEB81-F	TTACTCACTCAATACTTACCAATTC	Removing [-80, +221] from pGEB77
pGEB81-R	gtattgagtgagtaaCAAGCTTGCATGCCTG- CAGGTCGAC	Removing [-80, +221] from pGEB77
pGEB100-F	ATTTGATCGATCGAGGACAAGCTAG	Removing [+1, +301] from pGEB77
pGEB100-R	ctcgatcgatcaaatGAATGCAG- TTTCGGTGAATTCGCGTAATGGTACAT	Removing [+1, +301] from pGEB77
<b>Mutagenesis</b>		
pSW7848-F	GTCTGATTTCGTTACCAATTATGACAAC	vector amplification
pSW7848-R	GAATTCGATATCAAGCTTATCGATAC	vector amplification
Vsr217-up-F	aagcttgatcgaattcTCTTCCCAA- GACTTAGGTGGGTTAGGGACAAG	Upstream of <i>vsr217</i>
Vsr217-up-R	agtgagtaaGAATGCAGTTTCGGTGAATTCGCG- TAATGG	Upstream of <i>vsr217</i>
Vsr217-down-F	ctgcattcTTACTCACTCAATACTTACCAATTCC- TACAGTTACTGCTGAC	Downstream of <i>vsr217</i>
Vsr217-down-R	ttgtaacgaatcagacCCAGCAACGAAACGGTTTT- GAGGATAGTGG	Downstream of <i>vsr217</i>
malT-up-F	aagcttgatcgaattcTATTCGTAGTGAAGACCG- TAGCC	Upstream of <i>malT</i>
malT-up-R	cgaatcgtCTATTCCGTGGCGTATGTTTTG	Upstream of <i>malT</i>
malT-down-F	cggaatagACGATTCGAGATTGATTCG	Downstream of <i>malT</i>
malT-down-R	ttgtaacgaatcagacTAATGCTGTAAGCGAAGG	Downstream of <i>malT</i>
polyT up-F	ataagcttgatcgaattcAGACTTAGGTGGGTTAGG	Upstream of last 13nt of <i>vsr217</i>
polyT up-R	gagtgagtaaGGGACTTGAATTCTTTAACC	Upstream of last 13nt of <i>vsr217</i>
polyT down-F	ttcaagtcccTTACTCACTCAATACTTACCAATTC	Downstream of last 13nt of <i>vsr217</i>
polyT down-R	taattgtaacgaatcagacCAATCGTCGCATCACCTG	Downstream of last 13nt of <i>vsr217</i>
<b>sRNA interaction system</b>		
pZE12-F	CTAGAGGCATCAAATAAAACGAAAGGCTCA	pZE12MCS
pZE12-R	GGTACCTTTCTCCTCTTTAATGAATTCGGT	pZE12MCS
Vsr217-F	ttaaaggagaaaaggaccCCCTTTATCTACTCCTCCC	<i>vsr217</i>
Vsr217-R	gttttattgatgcctctagAACAGACAAAGCGCGATC	<i>vsr217</i>
pXG-10_fwd	GCTAGCAAAGGAGAAGAACTTTTC	pXG-10 (vector amplification)
pXG-10_rev	GTGCTCAGTATCTCTATCA	pXG-10 (vector amplification)
pZE-CAT	TGGGATATATCAACGGTGGT	pXG-10 (sequencing insert) (Urban & Vogel, 2007)
JV0-155	CCGTATGTAGCATCACCTTC	pXG-10 (sequencing insert) (Urban & Vogel, 2007)

Vibtas_FBP_UTR_ F	atagagatactgagcacATTACCATTAAAAA- GATCTAACCTTTAG	<i>fbp</i> 5' UTR [-125, +3]
Vibtas_FBP_UTR_ R_bis	agttcttctccttt- GCTAGCCATGTTATTTCCCTTAAACTTCTCTC	<i>fbp</i> 5' UTR [-125, +3]
Vibtas_GAP_UTR_ _F	tagagatactgagcacTTTATTTTTCCCTAGCAGAA- TAAC	<i>gapC</i> 5' UTR [-200, +3]
Vibtas_GAP_UTR_ _R_bis	agttcttctcctttgctagcCATTTTACTCGGGAC- CTTTCACGTT	<i>gapC</i> 5' UTR [-200, +3]

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**Table 3: Proteins whose level are affected by Vsr217 in maltose.**

Proteins involved in glycolysis/neoglucogenesis are indicated in red

Locus_tag	Fasta headers	Peptides	-log10 Welch's T- test p-value	log2 Fold change $\Delta$ vsWT in glucose	-log10 Welch's T-test p- value	log2 Fold change $\Delta$ vsWT in maltose
Upregulated proteins						
VS_II0899	prolyl aminopeptidase	10	0.0489858	-0.184973	2.72507	2.76785
VS_II0126	oxidoreductase	8	NaN	0	1.3286	2.54583
VS_II0346	glyceraldehyde-3-phosphate dehydrogenase GapC	18	0.0889369	0.144937	2.11486	1.73401
VS_II0148	catalase	10	NaN	0	1.34755	1.59973
VS_1054	histidine transport ATP-binding protein HisP	4	NaN	0	1.52598	1.59674
VS_1129	Signal transduction histidine kinase	4	NaN	0	2.05577	1.36148
VS_II1090	phosphomethylpyrimidine kinase	2	NaN	0	1.48825	1.26381
VS_2771	fructose-1,6-bisphosphatase Fbp	17	0.746935	0.211597	3.24572	1.23874
VS_II1225	phosphoenolpyruvate synthase PpsA	40	0.175205	0.276932	2.09341	1.07891
VS_2300	arsenate reductase	3	0.0118357	0.0449864	1.63985	1.06676
VS_II0877	MoxR-like ATPase	6	NaN	0	1.52205	1.00799
Down-regulated proteins						
VS_2926	ornithine carbamoyltransferase	17	0.121396	0.230532	1.37006	-1.02761
VS_2263	glutathione-regulated potassium-efflux system protein KefB	4	0.0796502	0.10221	1.56188	-1.04685
VS_II0953	nitrate reductase ; nitrate reductase large subunit	3	NaN	0	1.36334	-1.14437
VS_2312	arsenate reductase	4	NaN	0	1.37985	-1.15748
VS_II0935	ABC transporter substrate-binding protein	7	0.451213	0.507222	1.36588	-1.21731
VS_II0219	maltose/maltodextrin transporter ATP-binding protein MalK	23	0.988113	-1.08226	2.6606	-1.23689
VS_1158	hypothetical protein VS_1158	15	0.82412	1.00672	1.47303	-1.35927
VS_II0405	TRAP dicarboxylate transporter subunit DctP	14	0.537903	-0.569234	1.51771	-1.4176
VS_2935	sodium/solute symporter	3	NaN	0	1.40289	-2.09147
VS_0474	glutamate synthase [NADPH] large chain	64	0.409985	0.0313594	2.66545	-2.42065
VS_1157	histidine kinase	8	0.147159	-0.410522	3.45098	-2.9478

## Supplementary figure

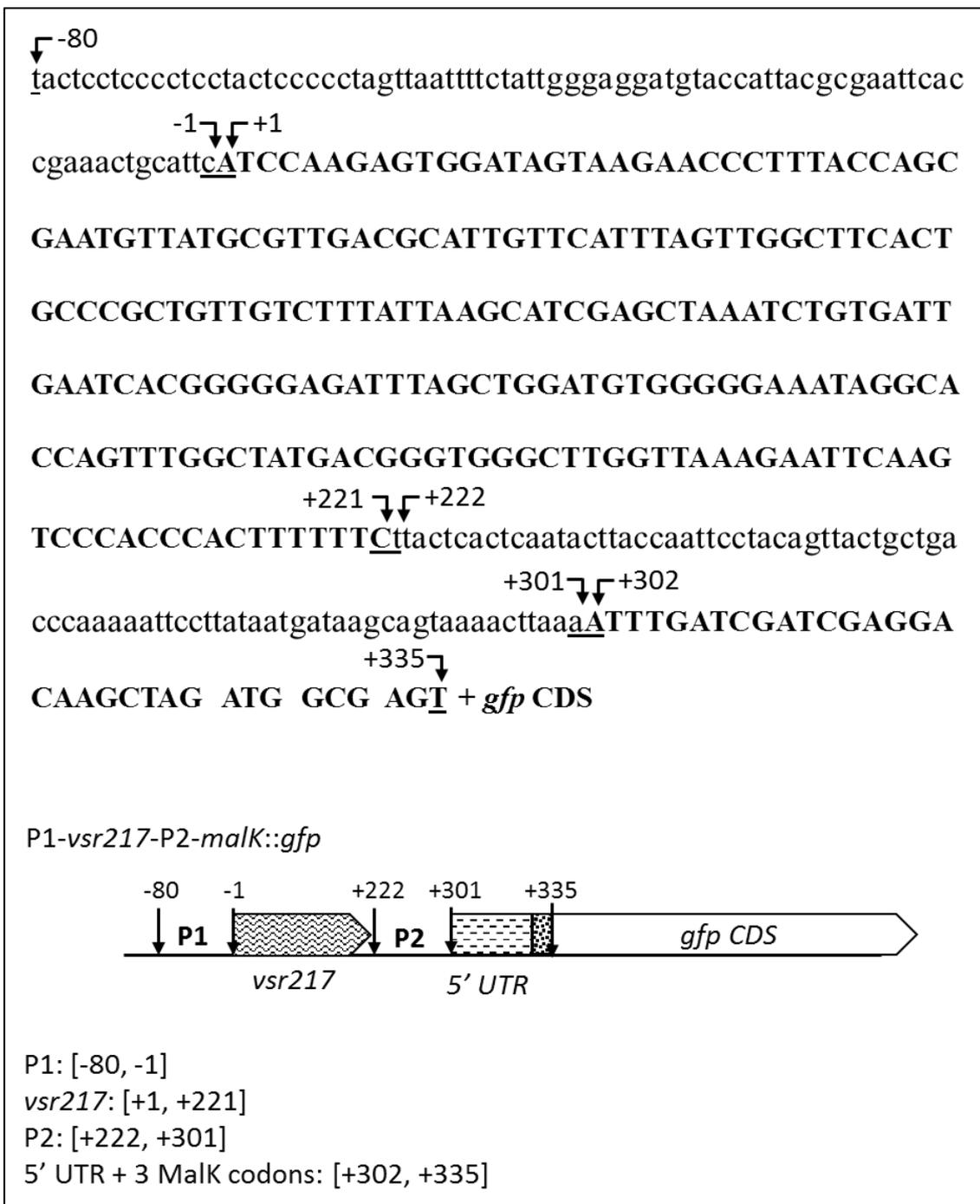


Fig. S1. Sequence of *vsr217* region showing coordinates of fragments used in the *gfpUV* fusion experiments



# GENERAL CONCLUSION AND PERSPECTIVES

In this thesis, I have presented the characterization of two *Vibrio* small regulatory RNAs: IlvL (chapter I) and Vsr217 (chapter II).

In both cases, these regulatory RNAs regulate important metabolic pathways. In *Vibrio*, most of the studied sRNAs are involved in the regulation of virulence, biofilm formation or quorum sensing (Table 3, Introduction). Only three sRNAs were described that are involved in the regulation of metabolism. In *V. cholerae*, the asRNA MtlS negatively regulates the expression of *mtlA* (encoding the mannitol transporter protein) by base pairing to the 5' UTR of the *mtlA* mRNA, occluding the RBS, and inhibiting the synthesis of the mannitol-specific phosphotransferase system system (Chang et al, 2015; Mustachio et al, 2012; Zhang & Liu, 2019). TarA, conserved in all *V. cholerae*, but not beyond, is under the control of the important virulence regulator ToxT, but negatively regulates the expression of *ptsG* (encoding a major glucose transporter) (Richard et al, 2010), providing an example of a possible link between virulence and metabolic adaptation. In *Aliivibrio salmonicida*, Spot 42 was shown to act as in *E. coli* and is involved in the regulation of motility and uptake of less preferred sugars (Hansen et al, 2012). Here, IlvL and Vsr217 are two additional regulatory RNAs who play a role controlling metabolic pathways. IlvL RNA is a *cis*-acting regulatory RNA belonging to a class that has been known for a long time, thanks to pioneering studies in the 1960s and 70s by Charles (Yanofsky, 2000), in the case of the tryptophan operon of *Escherichia coli*. This is the first report of such a mechanism in a *Vibrio* species which appears to be conserved in all *Vibrio* and beyond, highlighting the high conservation, and a probable ancient origin, of this mechanism in bacteria.

The case of Vsr217 is more novel and original: it is generated from a 5' UTR of *malK*, a gene involved in maltose transport, and is a member of the maltose regulon. We showed that Vsr217 negatively regulates the expression of two important enzymes involved in the glycolysis/neo-glucogenesis pathway, Fbp (fructose 1,6-bisphosphatase) and PpsA (phosphoenolpyruvate synthase). However, only *fbp* seems to be a direct target of Vsr217.

Because Vsr217 was highly induced in oysters, in which glycogen is a probable source of

maltose, we hypothesize that regulation of metabolism in presence of maltose provided by Vsr217 could be important for pathogenicity. However, the  $\Delta vsr217$  mutant did not show a decrease of virulence when injected in oysters, nor a decrease of cytotoxicity towards oyster hemocytes.

Both IlvL and Vsr217 are located in an mRNA leader region (Fig. 1). Such regulatory RNAs can be difficult to distinguish in RNA-seq data 5' UTRs and *trans*-acting regulatory RNAs encoded upstream of a gene transcribed in the same direction. In addition, it has been recently shown that bona-fide *cis*-acting sRNA can also act in *trans*. For instance, Melior *et al.* (Melior et al, 2019b) found that in *Sinorhizobium meliloti*, the tryptophan regulatory leader peptide RNA rTrpL is released under the condition of tryptophan sufficiency and can then base-pair with *trpD*, destabilizing the polycistronic *trpDC* mRNA (another tryptophan biosynthesis operon in this bacterium). This raised the possibility that IlvL RNA in vibrios could also play a role *in trans*, for example in regulating the *ilvIH* operon encoding a secondary acetolactate synthase. However, using IntaRNA, I could not detect interactions between IlvL and *ilvIH* RNA whereas I could identify another leader peptide upstream of *ilvIH*, indicating that the expression of *ilvIH* is probably not affected by IlvL but is regulated similarly to *ilvGMEDA*. More experiments would be necessary to confirm this.

In contrast, in the case of Vsr217, we have a new example of a growing class of sRNA generated from the 5' UTR of an mRNA. Only few 5' UTR-generated sRNAs have been published so far. In addition to the rTrpL mentioned above, 5' UTR generated sRNAs also include the S-adenosyl-methionine (SAM) riboswitches, SreA/B, which act both in *cis* to control their downstream genes upon binding SAM and in *trans* to target the virulence regulatory gene *prfA* mRNA in *L. monocytogenes* (Loh et al, 2009). Another example is Teg49 in *S. aureus*, a sRNA derived from the 5' UTR of *sarA* mRNA, that was reported to affect virulence independently (Kim et al, 2014; Manna et al, 2018a).

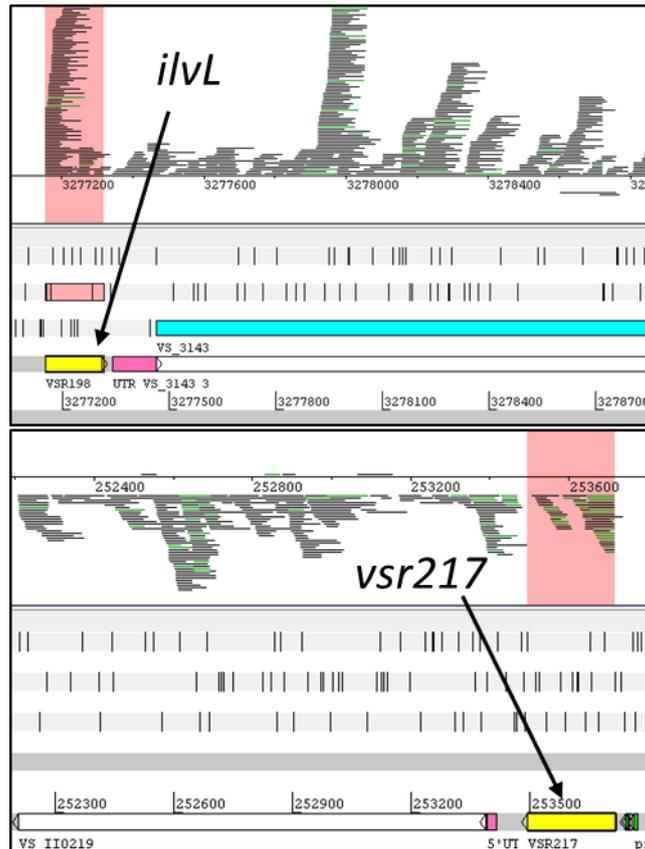


Fig. 1 An artemis window of *V. alginolyticus* *ilvL* RNA (top) and *V. tasmaniensis* *Vsr217* RNA (bottom)

Reads obtained by RNA-seq are mapped on the genome, and are represented by grey and green lines in the upper panel of each window. Bottom panel represents the annotated feature in the genome

Both *IlvL* and *Vsr217* show little primary sequence similarity to their homologous regions in other vibrios. Blast did not identify homologues beyond the closest species. Studying conservation of such RNAs required to use additional conserved features: conservation of secondary structures, neighboring genes and regulatory features. The alignment of the upstream region of *ilvG* gene in *V. alginolyticus* and *V. cholerae* (Fig. 9, Chapter I) showed a strong conservation of features of transcriptional attenuation: the presence of an ORF rich in codons for BCAAs followed by a highly conserved attenuator structure. Similarly, the alignment of the upstream region of *malK* gene in 7 vibrios (Fig. 1A&B, Chapter II) indicated that the primary sequence of the 5' and 3' domains and the secondary structure of *vsr217* are conserved among vibrios.

In addition, a MalT binding site was found in all of them. Importantly, the putative seed region of interaction of Vsr217 (Fig. 8C, Chapter II) is also conserved within a single stranded region of the secondary structure, allowing it to base-pair with its target. This is a common situation in most sRNAs, especially for those who regulate multiple targets via the same interaction site (Gottesman & Storz, 2011; Richter & Backofen, 2012; Storz et al, 2011), that the whole sequence shows low similarity while a short interaction site is well conserved.

In summary, Vsr217 represents a novel example of a sRNA that regulates a metabolic pathway as well as a member of a growing class of sRNA that are generated from 5' and 3' UTRs, contributing to the blurring the frontiers between established sRNA categories.

# RESUME DE LA THESE

Les bactéries occupent une grande variété d'habitats, où elles sont soumises à des changements constants de leur évolution : carences en éléments nutritifs, stress dû à la chaleur ou au froid, stress oxydatif, stress acide, lié à l'enveloppe cellulaire, l'exposition aux métaux lourds, un stress osmotique, etc. Pour survivre, elles doivent adapter leur physiologie à ces conditions par le biais de changements rapides et éventuellement dramatiques de l'expression des gènes. Chez les bactéries, la régulation des gènes s'effectue à différents niveaux : transcriptionnel, post-transcriptionnel et/ou traductionnel. La régulation transcriptionnelle par un facteur de transcription (TF), une protéine qui contrôle le taux de transcription en se liant à une séquence d'ADN spécifique, est connue de longue date (Browning & Busby, 2004). Plus récemment, l'importance des petits ARN régulateurs (ARNrég) dans divers processus cellulaires en tant que régulateurs post-transcriptionnels et traductionnels majeurs, en particulier en réponse à des changements de conditions de croissance et à des stress spécifiques, a été de plus en plus reconnue (De Mets et al, 2019; Durica- Mitic et al, 2018; Dutta et Srivastava, 2018; Frohlich & Gottesman, 2018; Kavita et al, 2018).

Les petits ARN régulateurs bactériens sont principalement des ARN non codants, de 50 à 500 nucléotides de long (nt). On a pu les identifier dans toutes les eubactéries et tous les archées où ils ont été recherchés (Wagner & Romby, 2015b). Ils peuvent agir en séquestrant des protéines ou en s'appariant avec des ARNm cibles, affectant ainsi leur traduction ou la stabilité de l'ARNm (Gottesman et Storz, 2011 ; Papenfort et Vogel, 2010 ; Storz et al, 2011; Wagner et Romby, 2015b).

Les *Vibrionaceae* constituent une famille de bactéries marines dont beaucoup sont responsables d'infections affectant non seulement les humains, mais également les poissons et les invertébrés marins, représentant la principale cause de mortalité chez les espèces marines d'élevage, et entraînant d'importantes pertes économiques. Ils sont capables de coloniser de nombreux habitats, où ils existent sous forme planctonique, de biofilms ou associés à divers hôtes. Cette

grande adaptabilité est liée à leur capacité à générer de la diversité génétique, en partie par le transfert latéral de gènes, mais également par divers mécanismes de régulations de l'expression génique. La régulation génique post-transcriptionnelle et traductionnelle médiée par les ARNrég a été étudiée principalement chez les entérobactéries. Les ARNrég de *Vibrio* sont beaucoup moins connus. En outre, la majorité des études sur les ARNrég dans les vibrions ont été réalisées sur *V. cholerae*, l'agent causal du choléra et se sont concentrées sur des ARNrég potentiellement impliqués dans la virulence et le Quorum sensing, qui lui-même régule la virulence. Dans cette thèse, je présente deux mécanismes de régulation distincts par deux ARNrég chez deux espèces différentes de *Vibrio* qui présentent deux spécificités : ils sont médiés par des ARNrég générés à partir des 5'UTR d'ARN messager, et ils régulent des voies métaboliques. La caractérisation de ces deux petits ARN font l'objet du chapitre I et du chapitre II respectivement. Le travail présenté dans le chapitre I fait l'objet d'une publication sous presse (Applied and Environmental Microbiology) et celui du chapitre II sera soumis prochainement.

# CHAPITRE I

## **L'opéron *ilvGMEDA* est régulé par l'atténuation de la transcription dans *Vibrio alginolyticus* ZJ-T**

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#: ces deux premiers auteurs ont également contribué à ce travail

\*: ces deux derniers auteurs ont également contribué à ce travail

*Applied and Environmental Microbiology* (sous presse)

En collaboration avec Yiqin Deng dans le laboratoire du Dr Chang Chen, j'ai exploré la régulation de la biosynthèse des acides aminés à chaîne ramifiée (BCAA- pour branched-chain amino acid en anglais : L-Leucine, L-Valine et L-Isoleucine) chez *V. alginolyticus*, un agent pathogène des poissons et crustacés de mer et un pathogène opportuniste émergent chez l'homme. Nous avons montré que chez cette espèce, l'opéron *ilvGMEDA* code la principale voie de biosynthèse des BCAA, les gènes *ilvGM* codant pour l'acétolactate synthase, un enzyme commun à la biosynthèse des trois amino acides. Un deuxième locus *ilvIH* code pour une deuxième acétolactate synthase secondaire, mais qui permet la survie d'un mutant  $\Delta ilvGM$  en absence de BCAA dans le milieu de croissance. Contrairement à *E. coli*, il semble qu'il n'existe pas de 3ème acétolactate synthase chez les vibrios.

La région amont régulatrice de *ilvGMEDA* ne montre aucune similarité de séquence avec la région correspondante d'*Escherichia coli* ou d'autres Enterobacteriaceae et, pourtant, nous avons pu démontrer que cet opéron est régulé par un mécanisme d'atténuation transcriptionnelle similaire à ce qui existe chez les entérobactéries. Il existe en amont de l'opéron *ilvGMEDA* un ARN<sub>rég</sub>, IlvL, qui code pour un peptide riche en codons BCAAs, dont la traduction, en réponse à la présence d'Ile, Leu et Val dans la cellule, permet la formation d'un terminateur de transcription, qui va bloquer l'expression des gènes aval.

En particulier, les expériences de northern blots que j'ai réalisés pour étudier l'expression des gènes *ilvG* et *ilvA* dans différents mutants ont permis de démontrer que :

1-La présence de BCAAs ou de l'un d'entre eux dans le milieu entraîne une diminution de l'expression de *ilvGMEDA*.

2- La délétion d'un terminateur rho indépendant situé entre *ilvL* et *ilvGMEDA* entraîne une forte expression constitutive de *ilvGMEDA*, qui devient insensible à présence des BCAAs.

3-Une délétion de l'ATG (codon d'initiation) de l'ORF codée par IlvL, qui prévient la traduction du peptide, entraîne une forte diminution de l'expression de *ilvGMEDA* et un défaut de croissance en absence de BCAAs, indiquant que la traduction de IlvL est indispensable pour permettre la formation d'une structure antiterminatrice et la transcription de l'opéron aval,

conformément au modèle proposé chez les entérobactéries (voir figure 8, chapitre I)

Cette étude est la première analyse détaillée de la régulation de la biosynthèse des BCAA chez une espèce de *Vibrio*. Elle met en évidence la conservation mécanistique de l'atténuation de la transcription malgré l'absence de conservation de la séquence primaire des régions régulatrices.

Ce travail est le résultat d'une collaboration du laboratoire avec celui du Prof Chang Chen en Chine. Outre ma participation à la conception des expériences et à l'analyse de l'ensemble des résultats, j'ai relayé toutes expériences de Northern blots après avoir construit les mutants nécessaires (délétion de l'atténuateur et du codon d'initiation).

## CHAPITRE II

**Un nouvel ARNreg du genre *Vibrio* appartenant au régulon maltose et  
généralisé à partir du 5'UTR de *malk* régule l'expression d'un enzyme majeur  
de la voie de la glycolyse**

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(Manuscrit en préparation)

*V. tasmaniensis* est un agent pathogène de l'huître, intracellulaire facultatif, capable de survivre et de se multiplier au sein des hémocytes, les cellules immunitaires de l'huître. Une étude transcriptomique antérieure de *V. tasmaniensis* LGP32 avait conduit à l'identification de centaines d'ARNs potentiels chez cette espèce. Parmi eux, il a été constaté que Vsr217 était conservé chez les vibrios. *vsr217* est situé en amont du gène *malK*, qui code une sous-unité du principal transporteur de maltose, et est fortement régulé à la hausse lors de l'infection des huîtres, comme l'a montré une étude transcriptomique plus récente de *V. tasmaniensis* infectant l'huître. Cette étude avait également montré que le métabolisme du maltose était fortement induit pendant l'infection, une observation qui pouvait s'expliquer par le fait que les huîtres sont riches en glycogène, un précurseur du maltose. Il nous a donc paru intéressant de caractériser plus précisément ce nouveau petit ARN.

J'ai montré que Vsr217 est induit par le maltose et régulée par MalT, le régulateur positif de tous les gènes du régulon maltose. Vsr217 est généré à partir de la région 5' UTR du message *malK* et j'ai pu mettre en évidence l'existence d'un messager *vsr217-malK*. La délétion de *vsr217* entraîne un défaut de croissance en présence de maltose comme source unique de carbone, en raison d'une réduction de MalK, liée à un effet en *cis* de la délétion, comme le démontre le fait que l'expression de MalK mais pas celle de Vsr217 en *trans* peut compléter le phénotype. Cet effet en *cis* peut s'expliquer par l'instabilité générée par la délétion de *vsr217* du messager *vsr217-malK*, source de 50% de l'expression de *malK*

J'ai également montré qu'un motif conservé à l'extrémité 3' de *vsr217* joue un rôle important dans la stabilité de Vsr217 sans affecter celle du messager *malK*. Le mutant délété pour ce motif ( $\Delta$ polyT), qui n'exprime pratiquement pas de Vsr217 ne présente en conséquence aucun défaut de croissance en présence de maltose comme seule source de carbone.

Ces résultats indiquaient que bien Vsr217 soit induit par le maltose, et fasse partie du régulon contrôlé par MalT, il ne joue aucun rôle apparent dans l'utilisation du maltose comme source de carbone. Nous avons donc émis l'hypothèse qu'il avait un rôle en *trans* pour réguler d'autres cibles.

Pour rechercher de telles cibles, nous avons utilisé une combinaison de méthodes expérimentales (protéomique) et bioinformatique (prédiction de cibles potentielles). Parmi les quelques gènes candidats, deux nous ont paru particulièrement intéressants. Il s'agit des gènes *fbp* et *ppsA*, deux gènes codant respectivement pour la fructose-1,6-bisphosphatase et la phosphoenolpyruvate synthase, deux enzymes importants de la voie de la néoglucogénèse/glycolyse. J'ai alors confirmé que l'absence de Vsr217 dans le mutant  $\Delta$ polyT conduisait à une augmentation des messagers de ces deux enzymes, suggérant que Vsr217 pouvait être un inhibiteur de leur expression.

L'utilisation de fusions traductionnelles entre la région 5' UTR de *fbp* et *ppsA* et le gène *gfp* utilisée comme gène rapporteur m'ont permis de montrer que ces deux gènes étaient des cibles directes de Vsr217, puisque l'expression des fusions est réprimée par l'expression de Vsr217 dans la souche *Escherichia coli*.

Fbp et PpsA sont les deux seules enzymes de la voie néoglucogénèse/glycolyse qui ne soient pas réversibles et travaillent uniquement dans le sens de la néoglucogénèse. Nous pouvons en conclure qu'en présence de maltose, l'induction de Vsr217 inhiberait la néoglucogénèse en diminuant l'expression de Fbp et PpsA, stimulant ainsi la glycolyse à partir du Glucose 6-phosphate généré par le catabolisme du glucose (Voir figure)

J'ai réalisé l'essentiel des expériences présentées dans ce chapitre, contribué à la conception expérimentale et à l'analyse des résultats. Ma directrice de thèse, Annick Jacq, a dirigé le travail, participé à la conception des expériences, analysé la conservation de Vsr217 (Fig. 1) et les résultats de l'analyse protéomique (Table 3). Philippe Bouloc a participé à la conception des expériences et à l'analyse des résultats.

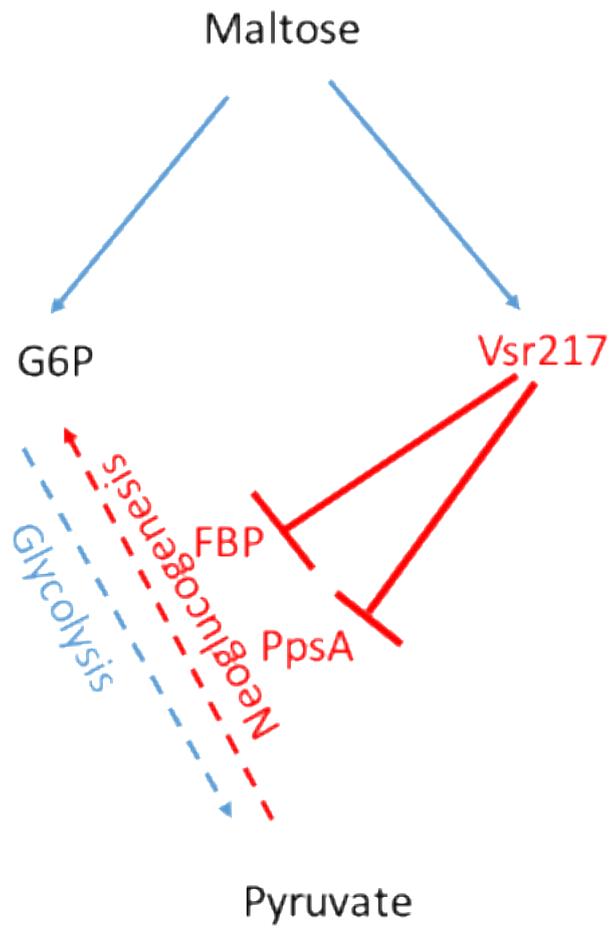


Fig 1. Représentation schématique du rôle de Vsr217 dans le métabolisme central de la cellule en présence de maltose comme source de carbone

## AUTRES TRAVAUX

### Les approches "dual RNA-seq" et métatranscriptomiques au service de l'étude des interactions hôtes-pathogènes

Les espèces de *Vibrio* causent des maladies infectieuses chez les humains et les animaux. Pour comprendre les mécanismes de pathogénèse, il est nécessaire d'interroger l'interaction entre l'hôte, son pathogène, et l'environnement. Cet environnement se caractérise par des facteurs abiotiques : température, pH, acidité, oxygène, etc, et des facteurs biotiques : nutriments fournis par les tissus, réponse immunitaire de l'hôte, mais aussi microflore commensale présente dans l'huître...

Les techniques de RNA-seq fournissent un outil très puissant pour analyser en parallèle les réponses transcriptionnelles de l'hôte et celles du pathogène. Des travaux antérieurs réalisés dans l'équipe de Delphine Detoumieux-Garzon, certains en collaboration avec notre équipe, ont montré l'importance de la réponse immunitaire de l'hôte et la capacité de se défendre du pathogène (réponse au stress oxydatif par exemple) pour déterminer l'issue du processus infectieux. Mais la façon dont *Vibrio* subvertit les défenses de l'hôte pour monter une infection réussie reste mal comprise, et cette connaissance est essentielle pour prédire et gérer la maladie. Malgré les progrès techniques réalisés, l'analyse d'un transcriptome bactérien à partir d'ARN totaux de l'hôte reste un challenge important. Pour y parvenir, j'ai développé un protocole qui intègre plusieurs kits pour éliminer efficacement les séquences d'ARNm des huîtres, d'ARN mitochondrial, d'ARN ribosomal et d'ARN ribosomal bactérien afin d'obtenir une profondeur de séquençage des ARN messagers et régulateurs bactériens suffisante pour permettre l'analyse. Les banques que j'ai ainsi préparées ont permis d'avoir accès au transcriptome de deux pathogènes, *V. tasmaniensis* et *V. crassostreae* pendant la colonisation de l'huître.

L'analyse en parallèle du transcriptome de l'huître réalisée par le laboratoire de Delphine Destoumieux-Garzon a montré l'importance de l'efficacité de la réponse immunitaire de l'hôte pour déterminer le succès ou non de l'infection. Des souches bactériennes proches mais non virulentes utilisées comme contrôles sont reconnues par le système immunitaire, et ne sont pas

capables d'échapper à cette réponse. Au contraire, les souches virulentes sont elles aussi reconnues, mais sont capables de neutraliser ces défenses de l'hôte, en particulier grâce à leur cytotoxicité pour les hémocytes. L'analyse du RNA-seq bactérien a permis de montrer que *V. crassostreae* et *V. tasmaniensis* utilisent des mécanismes distincts pour provoquer la lyse hémocytaire. Alors que la cytotoxicité de *V. crassostreae* dépend d'un contact direct avec les hémocytes et nécessite un gène ancestral codant pour une protéine de fonction inconnue, *r5.7*, la cytotoxicité de *V. tasmaniensis* dépend de la phagocytose et nécessite l'activation d'un système de sécrétion de type 6 dans les conditions intracellulaires.

Ces travaux ont fait l'objet d'une publication dans PNAS, présentée en annexe, dont je suis coauteur.

J'ai également mis à profit les protocoles de construction de banques RNA-seq enrichies en ARN bactériens à partir de l'hôte que j'ai mis au point, pour obtenir plus de quarante banques permettant l'analyse du métatranscriptome de l'huître malade en milieu naturel. Les données RNA-seq ainsi obtenues sont en cours d'analyse et permettront d'obtenir des indications précieuses sur l'influence du microbiote et de son activité dans les syndromes de mortalités dont sont victimes les huîtres française depuis une quinzaine d'années.

## DISCUSSION GENERALE

Les deux petits ARN régulateurs que j'ai étudiés dans ce travail de thèse, IlvL et Vsr217 se sont avérés réguler des voies métaboliques importantes, la biosynthèse des acides aminés branchés dans un cas, et la glycolyse dans le deuxième. Chez les vibrios, la plupart des ARNrég qui ont été étudiés sont impliqués dans la régulation de la virulence, la formation de biofilms ou la détection du quorum (Introduction, Tableau 3). Seuls trois petits ARN régulateurs impliqués dans la régulation du métabolisme ont été décrits. Chez *V. cholerae*, l'ARNm MtlS régule négativement l'expression de *mtlA* (codant pour la protéine transporteuse de mannitol) en s'appariant avec le 5' UTR de l'ARNm de *mtlA*, en inhibant la synthèse et inhibant la synthèse du système de la phosphotransférase spécifique du mannitol (Chang et al, 2015; Mustachio et al, 2012; Zhang et Liu, 2019). TarA, conservée chez tous les *V. cholerae*, mais pas au-delà, est sous le contrôle de l'important régulateur de virulence, ToxT, mais elle régule négativement l'expression de *ptsG* (codant pour un transporteur majeur du glucose) (Richard et al, 2010) suggérant un lien possible entre la virulence et l'adaptation métabolique. Chez *Aliivibrio salmonicida*, il a été démontré que Spot 42 agissait comme dans *E. coli* et est impliqué dans la régulation de la motilité et de l'absorption de sucres moins favorables (Hansen et al., 2012). Ici, IlvL et Vsr217 sont deux autres ARN régulateurs qui jouent un rôle de contrôle des voies métaboliques. IlvL RNA est un ARN régulateur à action cis appartenant à une classe connue de longue date, grâce aux études pionnières menées par Charles Yanofsky dans les années 1960 et 1970 (Yanofsky, 2000), dans le cas de l'opéron tryptophane d'*E. coli*. Il s'agit de la première mise en évidence d'un tel mécanisme chez une espèce de *Vibrio*, mécanisme qui semble être conservé dans tous les *Vibrio* et au-delà, soulignant la haute conservation et une origine ancienne probable de ce mécanisme chez les bactéries.

Le cas de Vsr217 est plus novateur et original : il est généré à partir du 5' UTR de *malk*, un gène impliqué dans le transport du maltose, et fait partie du régulon maltose. Nous avons montré que Vsr217 régulait en trans négativement l'expression de deux enzymes importantes de la voie de la glycolyse/néoglucogenèse, Fbp (fructose 1,6-bisphosphatase) et PpsA

(phosphoenolpyruvate synthase). Étant donné que Vsr217 est fortement induit dans les huîtres, où le glycogène est une source de maltose, nous nous sommes demandés si la régulation du métabolisme en présence de maltose fournie par Vsr217 pouvait être importante pour la pathogénicité. Cependant, le mutant *vsr217* n'a montré ni diminution de la virulence lors de l'injection dans les huîtres, ni diminution de la cytotoxicité envers les hémocytes d'huîtres (résultats non montrés). Cependant, il est fréquent que le phénotype de mutants d'ARNrég soit difficile à identifier, ces petits ARN agissant comme modulateurs fins de la régulation génique. Des expériences de compétition dans l'hôte du mutant avec le WT pourrait peut-être permettre de mettre en évidence une 'fitness' amoindrie pour le mutant.

IlvL et Vsr217 sont tous deux situés dans une région leader d'ARNm (Fig. 2). Dans le cas de IlvL, il agit en *cis*. Mais dans le cas de Vsr217, nous avons montré qu'il agit en *trans*, comme un ARNreg classique. Peu de petits ARN de ce type ont été identifiés. En effet, il peut être difficile de distinguer à partir de données RNA-seq de tels ARN régulateurs des ARN régulateurs agissant en *trans* codés en amont d'un gène transcrit dans la même direction. De plus, il a récemment été démontré que des petits ARNs agissant en *cis*, pouvaient également agir en *trans*. Par exemple, Melior et al. (Melior et al, 2019b) ont découvert que, dans *Sinorhizobium meliloti*, l'ARN *cis* codant pour le peptide régulateur du tryptophane rnTrpL est libéré dans des conditions d'abondance de tryptophane et peut alors s'apparier avec l'ARNm polycistronique *trpDC* (un autre opéron de biosynthèse de tryptophane dans cette bactérie), qui est ainsi déstabilisé. Cela suggère la possibilité que l'ARN IlvL dans les vibrions puisse également jouer un rôle en *trans*, par exemple dans la régulation de l'opéron *ilvIH* codant pour une acétolactate synthase secondaire. Cependant, nous n'avons pas pu détecter bioinformatiquement, des interactions entre IlvL et *ilvIH*, alors que nous avons détecté l'existence d'un autre peptide leader en amont de *ilvIH*, ce qui indique que l'expression de *ilvIH* n'est probablement pas affectée par IlvL mais qu'elle est régulée de manière similaire à *ilvGMEDA*. Plus d'expériences seraient nécessaires pour le confirmer.

En revanche, dans le cas de Vsr217, nous avons un nouvel exemple d'une classe croissante d'ARNs générée à partir du 5' UTR d'un ARNm. Jusqu'à présent, seuls quelques ARNs

généérés par 5' UTR ont été publiés. En plus de mTrpL mentionné ci-dessus, les ARNs agissant en trans et générés en 5' UTR incluent également les riboswitches S-adenosyl-méthionine (SAM), SreA/B, qui agissent à la fois en cis pour contrôler leurs gènes en aval suite à la liaison au SAM et en trans pour cibler la virulence. Le gène régulateur de l'ARNm de *prfA* chez *L. monocytogenes* (Loh et al, 2009) et Teg49 chez *S. aureus* sont d'autres exemples. Ce dernier est un petit ARN dérivé du 5' UTR de l'ARNm de *sarA*, codant pour un régulateur de la virulence, Teg49 affectant la virulence de manière indépendante de *sarA* (Kim et al, 2014; Manna et al, 2018a).

IlvL et Vsr217 présentent tous deux une faible similarité de séquence primaire avec leurs régions homologues dans d'autres vibrions. L'utilisation de Blast n'a pas permis d'identifier d'homologues au-delà des espèces les plus proches. L'étude de la conservation de tels ARN nécessite donc l'utilisation de caractéristiques conservées supplémentaires : conservation des structures secondaires, des gènes voisins et des caractéristiques de régulation. L'alignement de la région en amont du gène *ilvG* chez *V. alginolyticus* et *V. cholerae* (Fig. 9, Chapitre I) a mis en évidence une forte conservation des caractéristiques structurales d'un atténuateur transcriptionnel: la présence d'une petite ORF riche en codons pour les BCAA suivi d'un gène hautement conservé et d'une structure tige-boucle pouvant constituer l'atténuateur. De même, l'alignement de la région amont du gène *malk* dans 7 vibrions (Fig. 1A & B, Chapitre II) indique que la séquence primaire des domaines 5' et 3' et la structure secondaire de Vsr217 sont conservées. En outre, un site de liaison MalT a été trouvé dans chacun d'entre eux. Il est important de noter que la région supposée d'interaction de Vsr217 avec sa cible (figure 8C, chapitre II) est également conservée dans une région simple brin de la structure secondaire, ce qui permet l'appariement initial. Il est courant chez la plupart des petits ARNs, en particulier chez ceux qui régulent plusieurs cibles via le même site d'interaction (Gottesman et Storz, 2011; Richter et Backofen, 2012; Storz et al, 2011), que la séquence complète soit peu conservée alors que le site d'interaction initiale l'est.

En résumé, Vsr217 représente un nouvel exemple d'un petit ARN qui régule une voie métabolique, ainsi qu'un nouveau membre d'une classe croissante d'ARNs régulateurs générés

à partir de 5' et 3' UTR, contribuant à brouiller les frontières entre les catégories de petits ARN. Finalement, j'ai également réussi à mettre au point des protocoles de construction de banques RNA-seq permettant d'obtenir des transcriptomes bactériens à partir d'ARN totaux extraits d'huîtres, un outil puissant pour mieux comprendre les interactions hôtes-pathogènes et l'influence du microbiote commensal dans la résistance ou la sensibilité de l'huître aux pathogènes rencontrés dans l'environnement.

# ANNEX

## Species-specific mechanisms of cytotoxicity toward immune cells determine the successful outcome of *Vibrio* infections

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### Description

We have characterized cellular and molecular mechanisms leading to successful colonization in oysters infected with two virulent *Vibrio* species. I was involved in the work of within-host bacterial RNA-Seq (results shown in Fig. 5), which allowed us to know how these two vibrios reponse to host-imposed stresses. Specifically, I prepared 12 non-rRNA-bacterial RNA enriched libraries from total RNA obtained from oyster tissues. The challenge of this work was to enrich bacterial mRNA, which is a very small proportion, from total RNA. To achieve that, I developed a protocol which integrated several kits to effectively eliminate oyster mRNA, mitochondrial RNA, ribosomal RNA and bacterial ribosomal RNA sequences.



# Species-specific mechanisms of cytotoxicity toward immune cells determine the successful outcome of *Vibrio* infections

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***Vibrio* species cause infectious diseases in humans and animals, but they can also live as commensals within their host tissues. How *Vibrio* subverts the host defenses to mount a successful infection remains poorly understood, and this knowledge is critical for predicting and managing disease. Here, we have investigated the cellular and molecular mechanisms underpinning infection and colonization of 2 virulent *Vibrio* species in an ecologically relevant host model, oyster, to study interactions with marine *Vibrio* species. All *Vibrio* strains were recognized by the immune system, but only nonvirulent strains were controlled. We showed that virulent strains were cytotoxic to hemocytes, oyster immune cells. By analyzing host and bacterial transcriptional responses to infection, together with *Vibrio* gene knock-outs, we discovered that *Vibrio crassostreae* and *Vibrio tasmaniensis* use distinct mechanisms to cause hemocyte lysis. Whereas *V. crassostreae* cytotoxicity is dependent on a direct contact with hemocytes and requires an ancestral gene encoding a protein of unknown function, *r5.7*, *V. tasmaniensis* cytotoxicity is dependent on phagocytosis and requires intracellular secretion of T6SS effectors. We conclude that proliferation of commensal vibrios is controlled by the host immune system, preventing systemic infections in oysters, whereas the successful infection of virulent strains relies on *Vibrio* species-specific molecular determinants that converge to compromise host immune cell function, allowing evasion of the host immune system.**

T6SS | toxin | dual RNA-seq | cytolysis | pathogenesis

**V**ibrionaceae (hereafter vibrios) are  $\gamma$ -proteobacteria ubiquitous in oceans and marine coastal environments. They are free-living or associated with organic particles (sea snows) and organisms (1), in the latter case establishing a wide range of mutualistic, commensal, and pathogenic interactions with their hosts. The partnership between the luminous bacterium *Vibrio fischeri* and the Hawaiian bobtail squid *Euprymna scolopes* has been used to study mutualistic symbiosis in animals (2, 3). In contrast, some *Vibrio* species, including *Vibrio cholerae*, cause disease in humans (4). Other *Vibrio* species cause disease in wild and farmed marine animals including corals, shrimp, mollusks, and fishes, having significant ecological and economic consequences (5–7). In this context, the oyster *Crassostrea gigas* provides a model for studying the interactions of *Vibrio* species with an ecologically relevant host, specifically to understand their disease-causing properties (8).

In oysters, maintenance of homeostatic interactions between the microbiota and host immunity is poorly understood (9), and can be disrupted by abiotic and biotic factors, including pathogenic agents. For example, in oysters with Pacific oyster mortality syndrome, a *Herpes* virus was shown to cause immune

suppression, enabling microbiome dysbiosis and proliferation of bacteria such as *Vibrio crassostreae* (10). This species and other closely related species of the Splendidus clade (e.g., *Vibrio splendidus*, *Vibrio tasmaniensis*, and *Vibrio cyclitrophicus*) have been repeatedly isolated from diseased oysters, and their virulence confirmed in experimental oyster infections (11–14).

Most oyster–vibrio interactions have been studied by using a single strain, *V. tasmaniensis* LGP32, as a model. This strain is a facultative intracellular pathogen of hemocytes, oyster immune cells (15). Several LGP32 genes conferring resistance to reactive oxygen species (ROS) and heavy metals were shown to be required for protection against killing by hemocytes (16). Moreover, inside phagosomes, the LGP32 strain undergoes membrane remodeling

## Significance

***Vibrio* species are causal agents of important infectious diseases in humans and animals. Here, we have studied *Vibrio* infections in an ecologically relevant model host, oyster, for which natural virulent *Vibrio* strains are available. We have shown that virulent *Vibrio* species use different mechanisms to evade host cellular defenses. These mechanisms relied on distinct molecular determinants shared between strains of the same *Vibrio* species, but not between different species. The different mechanisms nonetheless converged on the same crucial outcome, immune cell lysis. Such cytotoxicity enabled virulent strains to cause systemic infections. Species-specific mechanisms that actively target host immunity could be more common than thought among *Vibrio* species, including human pathogens.**

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Genbank database (nucleotide accession nos. SEOP00000000, SESM00000000, SESL00000000, and VARQ00000000) and SRA database (BioProject accession no. PRJNA515169, SRA accession nos. SRR8551076–SRR8551093; BioProject accession no. PRJNA521688, SRA accession nos. SRR8567597–SRR8567602; BioProject accession no. PRJNA521693; SRA accession nos. SRR8573808–SRR8573813).

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that confers resistance to host antimicrobial peptides (17). This strain was shown to be cytotoxic to hemocytes (16), but the mechanisms causing cytotoxicity remain unknown, and it remains unclear whether other strains belonging to *V. tasmaniensis* or to the Splendidus clade as a whole are also intracellular pathogens and cytotoxic to hemocytes. Within the Splendidus clade, virulence is an ancestral trait that has been lost in several species (12). The ancestrally acquired *r5.7* gene, which encodes for an exported protein of unknown function, is necessary for virulence across the entire Splendidus clade (12, 14). The *r5.7* gene is absent from *V. tasmaniensis*, although this species includes virulent strains, suggesting that virulence relies, at least in part, on alternative mechanisms. Thus, to understand the basis of *Vibrio*-driven diseases, it is necessary to identify the specific determinants of virulence of the species belonging to the Splendidus clade and their potential host target(s).

An effective strategy to characterize different virulence pathways is to compare the genomes of closely related strains with potentially distinct virulence mechanisms. Complementary information can be obtained through global analyses of bacterial and host transcriptional responses, i.e., dual transcriptomics. Indeed, bacterial colonization has been shown to have substantial transcriptional effects on host and microorganisms. Thus, simultaneously measuring host and pathogen gene expression by using dual RNA-seq provides useful insights into the physiological changes that occur during the course of an infection, as well as the response of such changes to each other (18), which can be tested by using gene knock-out experiments.

In the present study, we have characterized the within-host cellular and molecular mechanisms leading to colonization success in oysters infected with 2 *Vibrio* species belonging to the Splendidus clade, *V. tasmaniensis* and *V. crassostreae*. By using an integrative approach, we provide a cellular and molecular description of the changes occurring in host and bacterial physiologies during the infection. Our results show that all virulent strains from both species actively repress host cellular defenses by killing hemocytes and colonize oyster tissues. We have found that the process of immune evasion and colonization is accompanied by the repression of a series of antibacterial defenses and manipulation of metal and ROS homeostasis. Interestingly, we have also identified distinct genetic determinants required for bacterial virulence and cytotoxicity in both vibrio species. Taken together, our results show that vibrios have acquired species-specific virulence mechanisms that converge in a common end, i.e., alteration in key cellular defenses in oysters.

## Results and Discussion

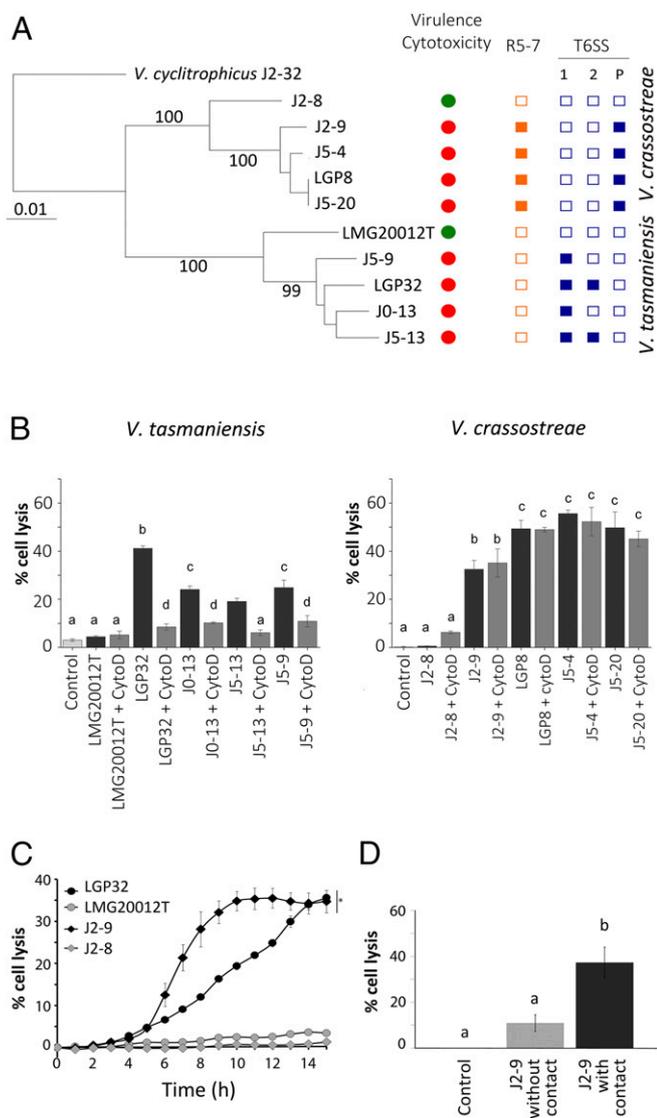
**Differential Cytotoxicity of Virulent *Vibrio* Strains.** Upon being phagocytized, the virulent *V. tasmaniensis* LGP32 strain causes high levels of hemocyte cytotoxicity (16). To determine if hemocyte cytotoxicity is a common feature of *V. tasmaniensis* and *V. crassostreae*, hemocytes were exposed to 4 virulent strains of each species, as well as a closely related but nonvirulent strain as a control (Fig. 1A). Only virulent strains induced hemocyte lysis (Fig. 1B). Nonetheless, the *V. crassostreae* J2-9 strain induced hemocyte lysis significantly more rapidly than the *V. tasmaniensis* LGP32 strain (Fig. 1C). Furthermore, treatment of hemocytes with cytochalasin D, which inhibits phagocytosis, strongly reduced the cytotoxicity of all *V. tasmaniensis* strains (RM-ANOVA  $P$  value < 0.001; Fig. 1B) but had no effect on *V. crassostreae* cytotoxicity. *V. crassostreae* J2-9 secretion products alone were unable to induce cytotoxicity (SI Appendix, Fig. S1), prompting us to ask whether cellular contact was required for J2-9 to kill hemocytes. Transwell experiments revealed that J2-9 cytotoxicity was significantly reduced in the absence of contact with hemocytes (RM-ANOVA  $P$  value < 0.01; Fig. 1D). Thus, although both species have major lytic effects on oyster hemocytes, their cytotoxicity is exerted through different mechanisms.

**Virulent Vibrios Escape Hemocyte Aggregation and Cause Systemic Infection.** To explore the pathogenesis of the virulent strains, we compared their histopathological effects versus those of nonvirulent strains. Strains were first injected into oysters from the same biparental family to reduce host genetic variability. Virulent strains (*V. tasmaniensis* LGP32 and *V. crassostreae* J2-9) induced mortality within 1 d and reached a plateau after 2–3 d (Fig. 2A). No significant differences were observed in the kinetics and rate of mortality induced by either of the strains, with ~40% of oysters surviving after 3 d (log-rank test,  $P = 0.802$ ; Fig. 2A). No mortalities were recorded in oysters injected with nonvirulent strains (LMG20012T, J2-8; Fig. 2A). The similarity in virulence of the 2 strains was confirmed in 2 other families of oysters, suggesting that virulence phenotypes do not depend on the genetic background of the oysters (SI Appendix, Fig. S2).

Bacterial load and tissue damages were analyzed 8 h after injection, before the first mortality occurred. Similar bacterial loads were observed for virulent and nonvirulent vibrios, with 1 copy of the vibrio genome per 25–50 copies of the oyster genome (SI Appendix, Table S1). However, localization of bacteria and damages to oyster tissues differed markedly between virulent and nonvirulent strains. Both virulent strains, LGP32 and J2-9, spread between muscle fibers and caused tissue disorder with loss of cellular integrity in the adductor muscle around the injection site (Fig. 2B). Moreover, a systemic and massive colonization of the conjunctive tissue was observed around the kidney, the digestive gland, the gonad, and under the basal lamina of the gut (Fig. 2B). In contrast, the nonvirulent strains LMG20012T and J2-8 did not cause any detectable tissue damage beyond the lesion caused by the needle injection in the muscle. These strains were not detected in histological sections or were restricted to the needle path within the adductor muscle (Fig. 2B). Instead, they were found in large hemocyte aggregates in the pericardial cavity, and, for J2-8, the kidney, aggregates that were not observed in oysters injected with virulent strains (Figs. 2B and 3A). These cell aggregates are reminiscent of extracellular DNA traps [referred to as Neutrophil extracellular traps (NETs) in mammals], which can bind and neutralize pathogenic bacteria and microorganisms, a defense mechanism conserved across species and recently observed in mollusks (19). In oysters, such DNA traps were shown to contain antimicrobial H1- and H5-like histones active against vibrios (20). Thus, our data suggest that virulent vibrios escape from hemocyte control and invade the oyster conjunctive tissue, whereas nonvirulent vibrios are contained within hemocyte aggregates, which we hypothesize prevent tissue colonization and associated tissue damage. In vivo, both virulent strains were highly cytotoxic to hemocytes. Indeed, *V. crassostreae* J2-9, but not the nonvirulent J2-8 strain, caused significant damage to circulating hemocytes within 8 h of oyster infection (Fig. 3B), consistent with previous observations for *V. tasmaniensis* LGP32 (16). In histological sections, *V. crassostreae* J2-9 was mainly observed outside hemocytes whereas *V. tasmaniensis* LGP32 and the nonvirulent strains J2-8 and LMG20012T were observed to have been heavily phagocytized (Fig. 3A). Therefore, our in vivo data are consistent with the cell contact-dependent and phagocytosis-dependent cytotoxicity observed in vitro for *V. crassostreae* and *V. tasmaniensis*, respectively (Fig. 1).

**Virulent Vibrios Bypass Host Defenses.** The outcome of an infection depends on a series of complex host and bacterial factors that coordinate and fine-tune host–pathogen interactions (21, 22). Herein, by implementing dual RNA-seq in an in vivo infection, we were able to analyze the mechanisms underpinning colonization success by vibrios in pathological and nonpathological contexts.

We focused on host responses by comparing the transcriptomes of oysters injected with vibrios (virulent strains LGP32 or J2-9 or nonvirulent strains LMG20012T or J2-8) versus that of mock-infected



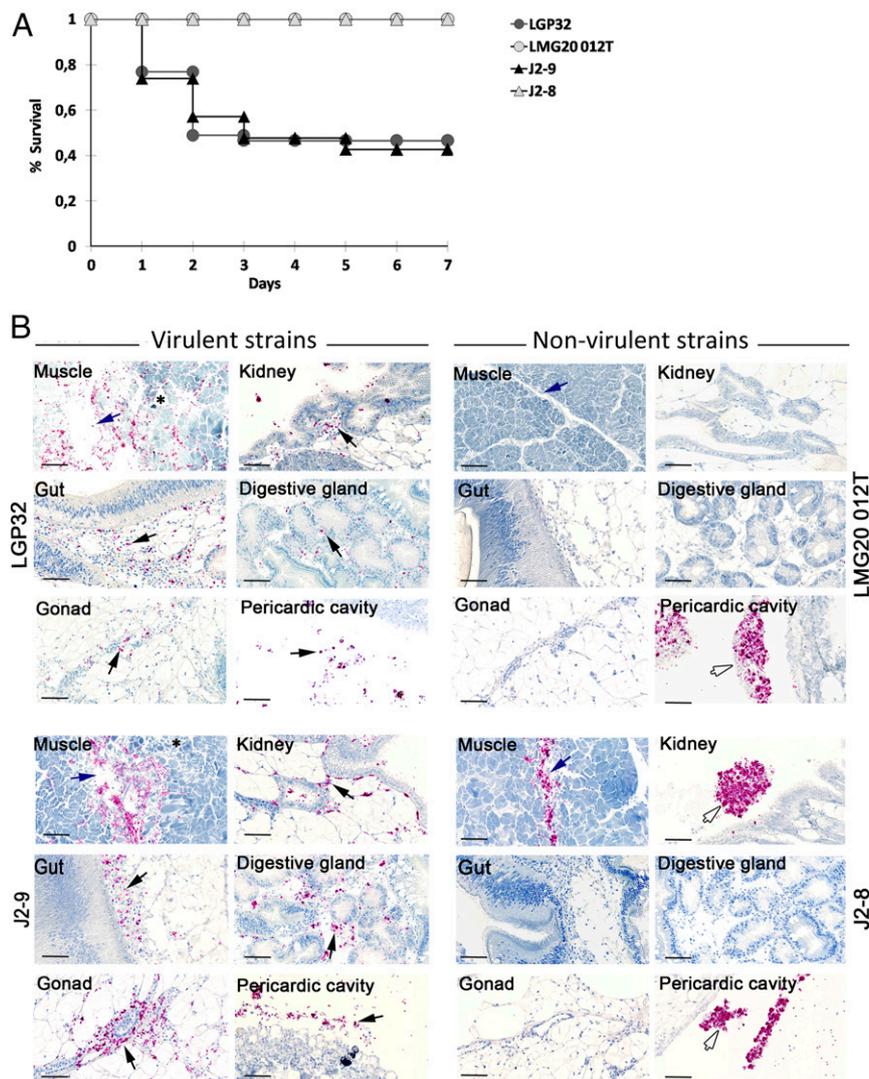
**Fig. 1.** Species-specific mechanisms of cytotoxicity in *V. crassostreae* and *V. tasmaniensis*. (A) Multilocus sequence typing tree of *V. crassostreae* and *V. tasmaniensis* with closely related nonvirulent strains. The tree was reconstructed with maximum-likelihood method from a concatenated alignment of 6 core genes (*gyrB*, *hsp60*, *mreB*, *recA*, *rpoD*, and *topA*) representing 10,626 sites. Node support was assessed with 100 bootstrap replicates. The strain cytotoxicity, which correlates with strain virulence (B), is indicated by a red circle, whereas absence of these phenotypes is indicated by a green circle. A filled orange square indicates the presence of the *r5.7* gene, which encodes for a known virulence factor. The effect of the presence of T6SS systems on the different replicons (1, on chromosome 1; 2, on chromosome 2; P, on a plasmid) is indicated by a filled blue square. (B–D) Cytotoxicity of vibrios on a monolayer of hemocytes was monitored by the SYTOX Green assay for 15 h. Cells were infected with bacteria at a multiplicity of infection (MOI) of 50:1. (B) Maximum cytotoxicity exerted by *V. tasmaniensis* or *V. crassostreae* strains in the presence (gray bars) or absence (black bars) of cytochalasin D, which was used as an inhibitor of phagocytosis. Error bars represent the SD of the mean (RM-ANOVA,  $P < 0.001$ ). All virulent strains are cytotoxic. Cytotoxicity appears to be dependent on phagocytosis for *V. tasmaniensis* virulent strains only. The cytotoxicity exerted by *V. crassostreae* was found to be higher than that of *V. tasmaniensis*. (C) Time-course analysis of cytotoxicity of J2-9 (black diamond), LGP32 (black circle), and LMG20 012T (gray circle) strains. J2-9 cytotoxicity is faster than LGP32 cytotoxicity. J2-8 and LMG20 012T are noncytotoxic. The error bar represents the SD of the mean (RM-ANOVA,  $P < 0.05$ ). (D) Maximum cytotoxicity for J2-9 strain was observed when in contact with hemocytes (black bars) vs. a Transwell plate without hemocyte contact (gray bars). The contact between hemocytes and vibrios is necessary for significant expression of J2-9 cytotoxicity. The error bar represents the SD of the mean (RM-ANOVA,  $P < 0.01$ ; SI Appendix, Fig. S1).

oysters (injected with sterile seawater). Oysters were killed 8 h after the infection and before the onset of mortality. From 3 independent experimental infections of the same family of oysters, we observed significant differential expression [false discovery rate (FDR)  $< 0.05$ ] for 331 host genes in response to vibrios (Dataset S1). RNA-seq data were validated by qRT-PCR (correlation  $R^2 = 0.92$ ; linear regression test,  $P < 0.001$ ; SI Appendix, Fig. S4).

All vibrios induced an immune response in oysters independently of their virulence. Among the 331 genes differentially expressed in response to vibrios, 129 responded similarly to all strains (Fig. 4A), with 25.6% of them being involved in immunity (Fig. 4B and Dataset S1). We observed an overrepresentation of transcripts from 3 major immune signaling pathways, the interleukin 17 (IL-17), tumor necrosis factor (TNF), and Toll-like receptor (TLR) pathways (Dataset S1). This includes transcripts homologous to TLR13 and TLR4, which have been shown to recognize bacterial 23S ribosomal RNA and LPS, respectively, in mammals (23, 24). IL-17 is also recognized as a key player in the immune response of oysters against bacterial infections (25). Consequently, all vibrio infections induced oyster antimicrobial defenses mainly based on antimicrobial peptides (big defensins) (26) (SI Appendix, Fig. S5), ROS (dual oxidase DUOX expression; Dataset S1), and heavy metals (metallothioneins and zinc transporters; Dataset S1). These antimicrobial functions are believed to control bacterial infections in oysters (10, 16) and more generally microbiome homeostasis across animal species (27, 28).

However, a set of shared antibacterial responses was specifically repressed in oysters infected by the virulent *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 strains, suggesting a common strategy of immune evasion. Overall, a much more extensive response was induced against infection by virulent vibrios (132 differentially expressed host genes) compared with that by nonvirulent strains (20 differentially expressed host genes; Fig. 4A). This response clustered according to vibrio virulence rather than their phylogenetic relatedness (Fig. 4C). Host responses were similarly altered by both virulent strains (57 genes; Fig. 4A) encompassing 4 main functional categories: metabolism, cell proliferation and differentiation, immunity, and extracellular remodeling (Fig. 4B and Dataset S1). Immune genes involved in antibacterial defenses were repressed (Dataset S1). These included the NADPH-mediated production of ROS, C-type lectin-mediated antibacterial immunity, and Deleted in Malignant Brain Tumors 1 (DMBT-1)-mediated mucosal immunity. We recently demonstrated the role that repression of ROS in oyster hemocytes plays in survival of *Vibrio tasmaniensis* (16). C-type lectin domain family 4 proteins, which display aggregation and direct antibacterial properties in oysters (29), are involved in the maintenance of microbiome homeostasis (30, 31). The DMBT-1 mucosal fluid protein functions in mucosal immunity, involving bacterial recognition and countering invasion (32), and has been shown to suppress the virulence of *P. aeruginosa* (33). These antibacterial defenses may play a role in controlling commensal bacteria, and their repression by virulent vibrios likely represents an important strategy to bypass the host defense mechanisms and avoid clearance.

Oyster responses to vibrios revealed the existence of virulence mechanisms specific to each species. *V. tasmaniensis* LGP32 infection was characterized by multiple specific host responses (48 genes) falling into 5 main categories: metabolism, stress response, cell proliferation and differentiation, metal homeostasis (mainly induced genes), and extracellular matrix remodeling (both induced and repressed genes; Fig. 4C and Dataset S1). The LGP32-specific modulation of genes belonging to “cytoskeleton dynamics” is consistent with the phagocytosis-mediated cytotoxicity of LGP32 (16). Likewise, specific induction of zinc transporters and metallothioneins revealed the involvement of “metal homeostasis” in LGP32 pathogenesis (Fig. 4C and Dataset S1). On the contrary, specific responses to *V. crassostreae* J2-9 (27 genes) mostly fell



**Fig. 2.** Virulent strains LGP32 and J2-9 colonize oyster connective tissues and cause damage to the adductor muscle (*SI Appendix, Fig. S2*). (A) Kaplan–Meier survival curves were generated from a biparental family of juvenile oysters (Decipher family 14) injected with the virulent LGP32 or J2-9 or the nonvirulent LMG20 012T or J2-8 ( $7.5 \times 10^7$  CFU per oyster). Mortalities were counted daily over 7 d in 2 tanks containing 20 oysters each. Only J2-9 and LGP32 induced significant mortalities in oysters. (B) Tissue localization and histopathology of oysters infected with vibrios. Juvenile oysters were injected with  $7.5 \times 10^7$  CFU of GFP-expressing LGP32, GFP-expressing J2-9, GFP-expressing LMG20012T, or GFP-expressing J2-8. After 8 h, oyster tissues were fixed and stained with hematoxylin. Vibrios were stained on histological sections with anti-GFP antibodies coupled to alkaline phosphatase (red staining). Photographs were taken with a NanoZoomer scanner. Virulent vibrios J2-9 and LGP32 were found in the pericardial cavity and in the connective tissue surrounding the kidney, the gut, the digestive gland, and the gonad (black arrow). Histological damage was observed in the adductor muscle near the site of injection, where virulent vibrios were abundant (blue arrow). No histological damage was recorded in oysters infected with nonvirulent strains. J2-8 was controlled by hemocyte aggregates in the muscle, the kidney, and the pericardial cavity, whereas LMG20012T was controlled by hemocyte aggregates in the pericardial cavity only (white arrow). (Scale bar: 20  $\mu$ m.)

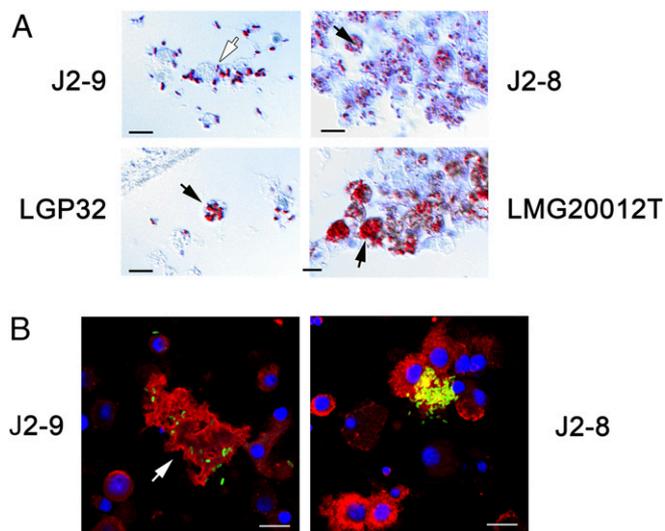
into the “immunity” category, representing 33% of the specific responses to J2-9 (Fig. 4 *B* and *C*). Immune genes were generally repressed, particularly those encoding C1q domain-containing (C1qDC) proteins (*Dataset S1*), a family of complement-related proteins, which, in oysters, can serve as opsonins that enhance vibrio phagocytosis (34). Their repression is consistent with the low levels of phagocytosis of *V. crassostreae* J2-9 (Fig. 3*A*). Genes in the category “cell signaling and adhesion” tended to be repressed by intracellular LGP32, whereas they were induced by J2-9. Thus, even though virulence appeared as the major criterion discriminating oyster responses to vibrios, specific signatures were also observed in the host response to distinct *Vibrio* species.

Overall, our oyster transcriptomics analyses suggest that the ability of virulent vibrios to escape hemocyte control and colonize oysters does not rely on the escape from immune recognition but

rather on an active repression of a set of antibacterial defenses. Moreover, we found that vibrio strategies to bypass host defenses rely, at least in part, on a species-specific alteration of host responses.

#### ***Vibrio* Gene Expression Support Species-Specific Virulence Mechanisms.**

Global changes in bacterial gene expression can be monitored to explore mechanisms driving colonization success during pathogenesis. We used bacterial RNA-seq to analyze the transcriptomes of virulent vibrios (LGP32 and J2-9) in infected oysters. *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 showed substantial transcriptomic reprogramming inside the host, with 46.7% (2,266 genes) and 50.1% (2,815 genes) differentially expressed genes, respectively, compared with time 0 (before injection; fold change  $\geq 2$ , FDR < 0.05; Fig. 5*A*). Overall within-host transcriptomes were comparable between the 2 virulent strains in terms of functional category distribution. A



**Fig. 3.** Virulent strains cause damage to hemocytes and escape hemocyte aggregates in vivo. (A) Juvenile oysters were injected with  $7.5 \times 10^7$  CFU of GFP-expressing LGP32, GFP-expressing J2-9, GFP-expressing LMG20012T, or GFP-expressing J2-8. After 8 h, oyster tissues were fixed and stained with hematoxylin. Photographs of hemocytes were taken in histological sections by using differential interference contrast (DIC) microscopy. Hemocytes and vibrios appear in blue and red, respectively. J2-9 is present inside and outside the hemocytes, whereas LGP32 and the nonvirulent vibrios are massively phagocytized. Hemocyte aggregates are observed with nonvirulent vibrios only. (Scale bar: 10  $\mu$ m.) (B) Confocal microscopy of hemolymph collected from animals infected with *V. crassostreae* J2-9 or the nonvirulent control J2-8. Juvenile oysters were injected with  $1.5 \times 10^8$  CFU of GFP-expressing vibrios. For each experimental condition, hemolymph was withdrawn from 5 oysters 2 h, 4 h, and 8 h after injection. Hemocytes were observed by confocal microscopy on Cyto-Spin glass slides after the staining of nuclei and actin cytoskeleton with DAPI and phalloidin-TRITC, respectively. In oysters injected with J2-9, J2-9 cells were often found associated with hemocyte lysis (mostly observed at 4 h and 8 h; white arrow), which was absent in J2-8-injected oysters at any time point. In contrast to J2-9, the nonvirulent J2-8 was massively phagocytized at 2 h and 4 h, often inside hemocyte aggregates; it became almost undetectable in the oyster hemolymph at 8 h, whereas J2-9 was still present (SI Appendix, Fig. S3). (Scale bar: 10  $\mu$ m.) Similar observations of in vivo hemocyte lysis have been recorded for LGP32 in a previous study (16).

common group of orthologous genes differentially expressed during oyster infection was identified, which encompassed 340 repressed and 501 induced genes (Fig. 5 B and C and Dataset S2). Most induced genes encoded proteins involved in “carbon compound and carbohydrate utilization” and “amino acid transport and metabolism” (Fig. 5C), reflecting a major metabolic shift in response to the host as a new environment (35). “Defense mechanisms” and “metal homeostasis” were also strongly induced in the *Vibrio* species, particularly genes encoding efflux machineries (multidrug, copper, and zinc transporters), siderophores, and iron uptake systems (Fig. 5C and Dataset S2). Therefore, gene expression of both virulent strains in oyster is consistent with a competitive behavior in a hostile environment that is poor in the essential iron but rich in zinc, copper, and other antimicrobials (Fig. 4C and SI Appendix, Fig. S4).

*V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 also displayed species-specific responses to colonization, which were mostly clearly observed when considering the 200 most highly induced genes of both strains (SI Appendix, Fig. S6). The *V. tasmaniensis* LGP32 response was characterized by a strong induction of genes involved in cell wall biogenesis, transport, regulation, and redox homeostasis (SI Appendix, Fig. S6). This result is in agreement with the known protective remodeling of vibrio membranes, and strong induction of antioxidants inside the phagosomes of oyster hemocytes

(16, 17). Several adhesins, including *mshM* (VS\_0329) and the type IV pilin assembly protein *pilB* (VS\_2548), were induced (Dataset S3), which may favor LGP32 attachment to hemocytes and promote phagocytosis, as previously shown for the major adhesin OmpU (15). Various expression patterns were observed for genes encoding putative RTX toxins. For example, the gene *VS\_II0512* was highly expressed inside and outside the host whereas *VS\_1394* was induced during colonization (Dataset S3). Oyster colonization had significant effects on the expression of genes encoding type 6 secretion systems (T6SS; reviewed in refs. 36–40) localized on the first (T6SS1) and second (T6SS2) chromosomes in *V. tasmaniensis*. T6SS is a multiprotein secretion system responsible for the transport and delivery of toxins (effectors) into recipient cells (39). It has been shown to contribute to pathogenicity toward plant and animal cells as well as to take part in interbacterial competition. T6SS2 genes were expressed only inside the host (Dataset S4), whereas T6SS1 genes were highly expressed outside the host and decreased to the levels of T6SS2 expression inside oysters (similar numbers of normalized counts; Dataset S4). The high preinfection expression levels of T6SS1 and the within-host expression of T6SS2 were confirmed over 24 h in oysters experimentally infected with *V. tasmaniensis* LGP32 (SI Appendix, Fig. S7).

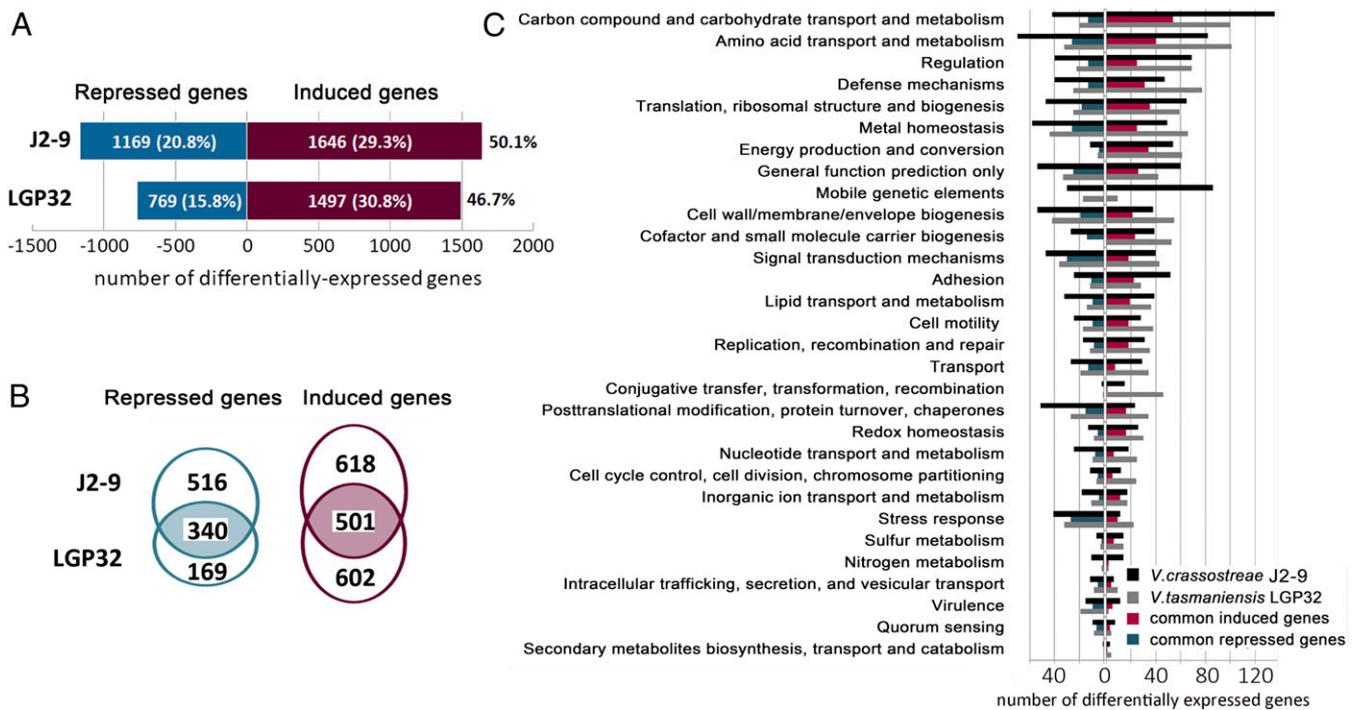
The response of *V. crassostreae* J2-9 to host colonization was characterized by a strong induction of adhesion genes (SI Appendix, Fig. S7), including a type IV pilus cluster, further supporting the role of attachment of the bacteria to the host target cells during the infection process (Dataset S2). The *r5.7* gene (VCR9J2v1\_730268) encoding an exported protein of unknown function, which has been previously shown to be necessary for virulence in *V. crassostreae* (12, 14), was one of the most highly induced virulence genes in J2-9 inside the host (Dataset S3). A number of genes encoding putative RTX toxins were highly expressed outside and inside the host, whereas other toxins and toxin secretion systems were expressed only inside the host (Dataset S3). In particular, a NAD-arginine ADP ribosyltransferase (putative toxin, VCR9J2v1\_730029) and 2 toxin secretion membrane fusion proteins (VCR9J2v1\_740022, VCR9J2v1\_740023) were expressed only inside the host (Dataset S3). Genes encoding a T6SS, carried by the virulence plasmid pGV1512 (11), were induced in oyster (Dataset S4). Genes related to the mobile genetic elements (Fig. 5C), particularly those involved in conjugative transfer, were very highly induced in J2-9 during oyster colonization (Dataset S2), suggesting that horizontal gene transfer between infecting *V. crassostreae* could occur during host colonization.

Taken together, cellular and molecular data highlight 2 strategies evolved by virulent vibrios in the Splendidus clade: *V. tasmaniensis* acts as an intracellular pathogen that subverts cellular functions and kills oyster phagocytes as part of its pathogenic process, whereas *V. crassostreae* acts as an extracellular pathogen that uses cell contact-dependent cytotoxicity to overcome the cellular defenses of its host. These results indicate that, within the Splendidus clade, distinct virulent species have acquired the capacity to compromise hemocyte functions to mount successful infections.

**Species-Specific Virulence Converges to Impair Host Defenses.** To confirm our conclusions that *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 use species-specific virulence determinants, we sought to identify genes responsible for virulence and cytotoxicity by using a gene knock-out approach. Candidate genes were selected based on their high expression or induction levels upon oyster colonization (Datasets S3 and S4). None of the mutants showed impaired bacterial growth in culture media (SI Appendix, Figs. S8A and S9A).

We knocked out potential adhesins and toxins. In *V. tasmaniensis* LGP32, the inactivation of an adhesin (*mshM*, VS\_0329), a type IV pilin assembly protein *pilB* (VS\_2548), and putative RTX toxins (VS\_II0512 and VS\_1394) did not significantly attenuate virulence in the experimental infection of oysters (SI Appendix, Fig. S8B).





**Fig. 5.** Responses to host-imposed stresses revealed by within-host bacterial RNA-seq. Within-host transcriptomes of *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 (8 h after infection) were compared with bacterial transcriptomes before the infection (controls) in 3 independent experiments. Only those genes whose expression varied significantly by more than 2-fold were considered. (A) Number of bacterial genes significantly induced and repressed during host colonization. The proportion of differentially expressed genes over the entire transcriptome of LGP32 and J2-9 is given as a percentage of the total number of genes for each genome. Approximately half of the total bacterial transcriptome is altered during host colonization. Repressed and induced genes are shown in blue and purple, respectively. (B) Venn diagram showing the core response of pathogens during colonization (340 repressed genes and 501 induced genes, not including genes with an unknown function). The diagram was built on common orthologous genes differentially expressed upon host colonization in J2-9 and LGP32. (C) Functional distribution of the bacterial genes during LGP32 and J2-9 colonization process (genes without annotated function were not included; *SI Appendix*, Figs. S6 and S7 and Dataset S2).

host cells (45). Together, these examples indicate that phagocytes are a natural target for T6SS in *Vibrio* species. They demonstrate that T6SS functions to repress immune defenses in different hosts during critical intracellular stages of infection. Like in other Gram-negative bacteria adopting intracellular stages, we show here that T6SS plays a critical role in the success of vibrio infections (42, 43).

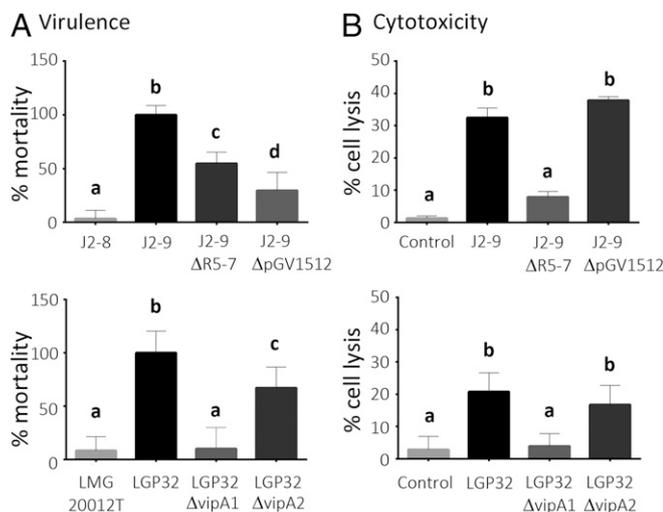
T6SS2 in *V. tasmaniensis* LGP32 was induced upon oyster colonization and reached maximum expression in dead oysters (*SI Appendix*, Fig. S7). However, this T6SS2 is absent from 2 other *V. tasmaniensis* virulent strains (J5-9 and J0-13; Fig. 1A and Dataset S4). Inactivation of T6SS2 did not alter LGP32 virulence and cytotoxicity (Fig. 6), indicating that it does not have a direct effect on host-pathogen interactions. Closer examination of the T6SS2 gene cluster revealed the absence of an evolved *vgrG* gene and the lack of a *paar* gene in proximity to *vgrG*. However, 7 genes encoding PAAR proteins and putative AHH endonucleases were present in tandem in a proximal region of the T6SS2 (*SI Appendix*, Figs. S10B and S11). This suggests that T6SS2 may play a role in interbacterial competition during colonization of oyster tissues, as shown for *V. fischeri* in its mutualistic association with squid (46). It could also be needed to exploit host resources after host death.

In *V. crassostreae* J2-9, the single T6SS was induced upon host colonization. This T6SS, carried by the plasmid pGV1512, is present in all *V. crassostreae* virulent strains (Fig. 1A and Dataset S4). As the encoded PAAR and VgrG proteins lack C-terminal effector domains (*SI Appendix*, Figs. S10 and S11), and because we were unable to identify any putative T6SS effector, the function of T6SS in *V. crassostreae* cannot presently be predicted. Even though it has previously been demonstrated that pGV1512 is necessary for J2-9 virulence (11), we did not observe any alteration

in the cytotoxicity of J2-9 upon the loss of pGV1512 (Fig. 6B). Therefore, we have no evidence that T6SS could be involved in *V. crassostreae* cytotoxicity toward oyster hemocytes. Instead, one of the most highly induced J2-9 virulence genes inside the host, *r5.7* (Mage VCR9J2v1\_730268; GenBank accession no. WP\_048663858.1), was demonstrated to be necessary for cytotoxicity ( $P < 0.001$ ; Fig. 6), and was also required for cytotoxicity in *V. crassostreae* strain J5-5 (*SI Appendix*, Fig. S13). Previous work has shown that *r5.7* alone is not toxic toward oysters and suggested that the protein interacts with the external surface of *Vibrio* and/or its cellular targets in oysters (12). *r5.7* is a virulence gene widely distributed across the Splendidus clade, and we show here that it is a key determinant of *V. crassostreae* cell contact-dependent cytotoxicity to hemocytes, yet has been lost in *V. tasmaniensis* species. Thus, our results show that, in virulent species of the Splendidus clade in which the ancestral virulence gene *r5.7* has been lost, other mechanisms have been acquired (T6SS1 in *V. tasmaniensis*) that specifically target immune cells through cytotoxic mechanisms that enable successful host colonization.

### Conclusion

Although cytotoxicity is widespread among *Vibrio* species pathogenic for humans and animals and is known to be important in disease progression, it has not been clearly associated with immune repression. Here, we demonstrate that the fate of vibrio infections in oysters, a natural host for marine *Vibrio* species, is determined by the specific mechanisms that have evolved in virulent strains to induce cytotoxicity. These mechanisms impair the host cellular defenses that normally prevent vibrios from causing systemic colonization of host tissues. Cytotoxicity toward immune cells was shown to be



**Fig. 6.** Species-specific virulence factors are needed for immune cell lysis. (A) Virulence of *V. crassostreae* (Upper) and *V. tasmaniensis* (Lower) isogenic mutants was assessed by intramuscular injection of juvenile oysters. Mortalities were recorded at day 1. Data are expressed as percentage of mortality induced by the wild-type strains. (B) Cytotoxicity of vibrio isogenic mutants lacking virulence genes was determined on monolayers of hemocytes and monitored by the SYTOX Green assay. Cells were infected with bacteria at an MOI of 50:1. Maximum cytotoxicity displayed by vibrios is shown here. Error bars represent the SD of the mean (RM-ANOVA,  $P < 0.001$ ; *SI Appendix, Figs. S8 and S9*).

dependent on distinct molecular determinants present in *V. crassostreae* and *V. tasmaniensis*, and other virulent species of *Vibrio* have been shown to be equipped with different specific virulence mechanisms. For example, in the Splendidus clade, MARTX toxins are found exclusively in *V. splendidus* and are necessary for virulence toward oysters (12). In more distant species from the Anguillarum clade, *Vibrio aestuarianus* uses a metalloprotease to evade hemocyte control (47, 48) and cause septicemia in oysters (49). Thus, *Vibrio* species that are pathogenic for oysters have evolved a diverse array of molecular mechanisms that all converge on the attenuation of the cellular defenses of their host, enabling subsequent tissue colonization (Fig. 7). As these mechanisms have been acquired independently during evolution in diverse species pathogenic for oysters, they could be a good indicator of the adaptation of these pathogenic species to oysters as a niche (50). Beyond oyster pathogens, a specific mechanism of phagocyte targeting has also been reported in *V. cholerae* (45). We believe more attention should be paid to *Vibrio* cytotoxicity in repressing host immunity, as this may be an insufficiently explored determinant of infection outcomes in metazoan hosts.

## Materials and Methods

**Strains, Plasmids, and Primers.** All strains, plasmids and primers used or constructed in the present study are described in *SI Appendix, SI Materials and Methods and Tables S6–S8*.

**Animals.** *C. gigas* diploid oyster spat were used from 3 families of siblings (Decipher 1, 14, and 15) (10), and a batch of standardized Ifremer spats (NSI) were produced from a pool of 120 genitors (51). Spats were used for experimental infections and dual RNA-seq. Adults produced from a pool of 120 genitors (AS1) were used to collect hemolymph for cytotoxicity assays (*SI Appendix, SI Materials and Methods*).

**Experimental Infections.** Experimental infections were performed at 20 °C as in ref. 52 by injection at  $7.5 \times 10^7$  CFU into oyster adductor muscle (details in *SI Appendix, SI Materials and Methods*). Statistical analyses were performed by using a nonparametric Kaplan–Meier test to estimate log-rank values for comparing conditions (GraphPad Prism 6).

**Molecular Microbiology.** *Vibrio* isolates were cultured as described in *SI Appendix, SI Materials and Methods*. Conjugation between *Escherichia coli* and *Vibrio* were performed at 30 °C as described previously (53). To label the strains with a fluorochrome, *gfp* gene was cloned in Apa1/Xho1 sites of the pMRB plasmid known to be stable in *Vibrio* spp. (54), resulting in a constitutive expression from a  $P_{lac}$  promoter. Gene inactivation was performed by cloning approximately 500 bp of the target gene in pSW23T (55) and selecting on Cm the suicide plasmid integration obtained by a single recombination (56). Mutants were screened for insertion of the suicide vector by PCR by using external primers flanking the different targeted genes (*SI Appendix, Table S2*).

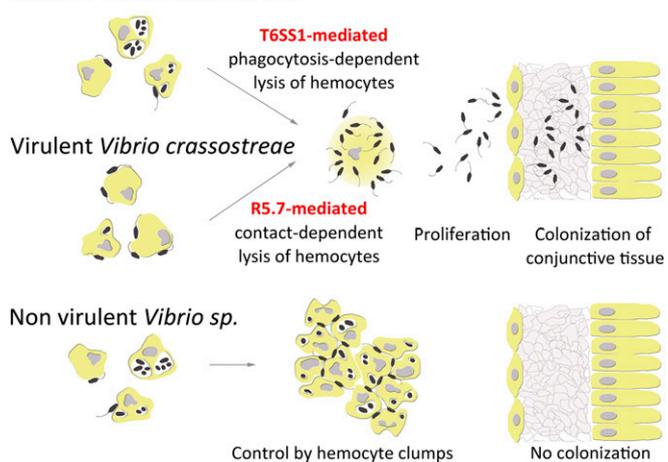
**Histology.** Spats (1.5–2 cm) were injected with  $7.5 \times 10^7$  CFU of GFP-expressing LGP32, J2-9, LMG20 012T, or J2-8. Noninjected oysters were used as controls. Spats were kept in seawater tanks at 20 °C, sampled, and fixed 8 h after injection. Histological sectioning was performed at HISTALIM (Montpellier, France). Longitudinal sections were immunostained with an anti-GFP primary antibody coupled to alkaline phosphatase (Abcam). Sections and hemocytes were observed as described in *SI Appendix, SI Materials and Methods*.

**In Vitro Cytotoxicity Assays.** Cytotoxicity of vibrios was measured on hemocyte monolayers according to procedures described in *SI Appendix, SI Materials and Methods*. Statistical analysis was performed by using RM-ANOVA.

**Confocal Microscopy of Interaction Between Hemocytes and Vibrios in Vivo.** Oysters were injected with  $1.5 \times 10^8$  CFU of GFP-expressing vibrios (J2-8 or J2-9) a priori washed and resuspended in sterile seawater. Control oysters received an injection of an equal volume of sterile seawater. For each experimental condition, hemolymph was collected from 5 oysters at 2 h, 4 h, and 8 h after injection. Samples were fixed with 4% paraformaldehyde and stained with DAPI and Phalloidin-TRITC and observed by confocal microscopy as described in *SI Appendix, SI Materials and Methods*.

**Dual RNA-Seq Experiments.** 3 independent experimental infections were performed. For every experiment, 150 oysters (30 animals per condition) were injected with bacteria ( $10^7$  CFU of LGP32, LMG20012T, J2-9, or J2-8) or sterile seawater (mock-infected oysters). In addition, both bacterial suspensions and 30 nontreated oysters were collected at time 0. For every experimental

## Virulent *Vibrio tasmaniensis*



**Fig. 7.** Impairment of oyster cellular immunity as a common strategy of immune evasion in vibrios. Virulent strains of the species *V. tasmaniensis* and *V. crassostreae* exert cytotoxic activity toward immune cells. They thereby repress key cellular defenses, gain access to the conjunctive tissues, and cause systemic infection. In *V. crassostreae*, cytotoxicity is a rapid, contact-dependent phenotype that requires the R5.7 virulence factor, which is strongly induced inside the host. In *V. tasmaniensis*, which behaves as an intracellular pathogen, cytotoxicity is phagocytosis-dependent and requires the intracellular action of a constitutively expressed type 6 secretion system present on chromosome I. In contrast to virulent strains, nonvirulent strains of both species are controlled by hemocyte clumps in the hemolymph compartment, preventing them from causing systemic infection. We conclude that the outcome of the infection depends on independently evolved molecular determinants that impair oyster cellular defenses as a strategy of immune evasion.

condition, 30 oysters were then sampled 8 h after injection (i.e., before mortality occurred) before RNA extraction (*SI Appendix, SI Materials and Methods*). For oyster RNA-seq, library construction and sequencing were performed on polyadenylated mRNAs by Fasteris (Geneva, Switzerland; <https://www.fasteris.com>). Directional cDNA libraries were constructed and sequenced on an Illumina HiSeq system in paired-end reads of  $2 \times 75$  bp. For bacterial RNA-seq, total RNA obtained from oyster tissues was used to prepare non-rRNA-bacterial RNA enriched libraries. After polyadenylated mRNAs (i.e., host mRNAs) were removed by using a MICROBEnrich Kit (Ambion), cDNA-oriented sequencing libraries were prepared starting from 100 ng enriched RNA by using an Ovation Universal RNA-Seq System kit (NuGEN). Library construction included a depletion step of abundant, nondesired sequences by using a Nugen-customized mixture of primers complementary to RNA sequences to be depleted, viz. oyster nuclear, mitochondrial, and ribosomal and bacterial ribosomal RNA sequences. The libraries were quantified by DeNovix dsDNA Broad Range Assay (DeNovix), and the quality was monitored by using an Agilent High Sensitivity DNA kit (Agilent Technologies). For each condition, 3 biological replicate libraries were prepared. Libraries were sequenced in paired-end mode ( $2 \times 150$  bp) by Fasteris on an Illumina HiSeq 4000 system to obtain ~100 million reads per sample per run.

**Oyster Transcriptome Analysis.** Data were treated using the galaxy instance of the IHPE laboratory (57). Phred quality scores were checked by using the FastX toolkit galaxy interface ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)) and were greater than 26 for more than 90% of the read length for all of the sequences. All of the reads were thus kept for subsequent analyses. Mapping to the *C. gigas* reference genome [assembly version V9 (58)] was performed by using RNA STAR (Galaxy version 2.4.0d-2; ref. 59). HTSeq-count was used to count the number of reads overlapping annotated genes (mode Union; Galaxy Version v0.6.1) (60). The resulting files were used as input to determine differential expression for each feature between oysters injected with vibrios and mock-infected oysters by using DESeq2 (61). Fold changes in gene expression between test animals and controls were considered significant when the adjusted *P* value ( $P_{adj}$ ) for multiple testing with the Benjamini–Hochberg procedure, which controls FDR, was  $<0.05$ . The same threshold was applied to fold changes in gene expression between mock-infected oysters and naïve controls. As genes for *C. gigas* antimicrobial peptides (AMPs) are not annotated in the *C. gigas* reference genome (assembly version V9; ref. 58), read counts for all AMPs were obtained by alignment to an in-house database of AMP protein sequences by using DIAMOND 0.7.9 (62) as described in *SI Appendix, SI Materials and Methods*. Oyster RNA-seq data have been made available through the SRA database (BioProject accession no. PRJNA515169) under SRA accession nos. SRR8551076–SRR8551093 (63).

**Vibrio Transcriptome Analysis.** Raw Illumina sequencing reads were trimmed by Trimmomatic in paired-end mode (64) to remove sequences corresponding to the NuGEN-provided adapters, clipping part of reads having a quality score of below 20, and dropping reads of less than 36 nt. The 2 resulting files were used as input for Bowtie2 (65) to align the reads on *V. tasmaniensis* LGP32 or *V. crassostreae* J2-9 genome, respectively (*SI Appendix, SI Materials and Methods*). As the rate of read alignment on the bacterial genome was ~2% for vibrios in oyster tissues, as opposed to >98% for the in vitro grown bacteria, we introduced a random down-sampling step of the aligned reads (<http://broadinstitute.github.io/picard/command-line-overview.html#DownsampleSam>; *SI Appendix, SI Materials and Methods*). Counts for each annotated feature (CDS + sRNAs) of *V. tasmaniensis* LGP32 or *V. crassostreae* J2-9 were then computed by using featureCounts (66). Differential expression was determined for each feature between oysters injected with vibrios and vibrios grown in vitro by DESeq2 (61). Genes were considered as differentially expressed between tested conditions and controls when  $|\log_2(\text{fold change})| \geq 1$  and  $P_{adj}$  for multiple testing with the Benjamini–Hochberg procedure was  $<0.05$ . Bacterial RNA-seq data have been made available through the SRA database: BioProject accession no. PRJNA521688 under SRA accession nos. SRR8567597–SRR8567602 (*V. crassostreae* J2-9) (67) and BioProject accession no. PRJNA521693 under SRA accession nos. SRR8573808–SRR8573813 (*V. tasmaniensis* LGP32) (68).

**Functional Gene Ontology Annotations.** For oyster genes, Blastx (69) comparison against NR database was performed for each set of genes with a maximum number of target hits of 20 and a minimal e-value of 0.001. XML blast result files were loaded onto Blast2GO (70) for GO mapping and annotation of the B2G database. Functional categories corresponding to significantly modulated genes (331 genes) were then determined manually; this was supported by background literature review. A heat map of the mean of  $\log_2$  fold change in 3 independent experiments was then generated by using cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>). For bacterial genes, annotations of differentially expressed genes (fold change  $\geq 2$  and  $P_{adj} \leq 0.05$ ) were manually curated by using the MicroScope platform (71) and the MetaCyc database (72). Based on these curated annotations, each gene was assigned to 1 of 32 functional categories (the list of categories is provided in Fig. 5 and *SI Appendix, Fig. S6*). The MicroScope tools were used to compare genomes based on sequence and synteny conservation (*SI Appendix, SI Materials and Methods*).

**RT-qPCR.** RT-qPCR was performed on the same RNA samples as those used for deep sequencing to validate RNA-seq data (*SI Appendix, Fig. S4*) or on specific RNA samples to monitor the time course of bacterial gene expression in oysters (*SI Appendix, Fig. S7*). cDNA synthesis and real-time qPCR were performed as described in *SI Appendix, SI Materials and Methods*.

**Specific Vibrio Quantification by qPCR.** Genomic DNA was extracted with the DNA kit Promega (Wizard SV Genomic DNA Purification System) on the same oyster samples used for RNA-seq. Real-time qPCR was used to amplify monocopy genes from specific vibrio genomes (*SI Appendix, Table S2*). To quantify oyster genomes, Cg-BPI (GenBank accession no. AY165040) primers were used (*SI Appendix, Table S2*). The vibrio charge in oyster tissues was calculated as a number of vibrio genome copies per number of oyster genome copy (*SI Appendix, SI Material and Methods*).

**Vibrio Genome Sequencing and Assembly.** Genomic DNA for J0-13, J5-13, and J5-9 was sequenced at the Bioenvironment platform (University of Perpignan, Cemeb) by using a NextSeq 550 instrument resulting in ~100-fold coverage (*SI Appendix, SI Material and Methods*). Reads were assembled de novo by using Spades software. Computational prediction of coding sequences together with functional assignments was performed by using the automated annotation pipeline implemented in the MicroScope platform (73). The genome sequences of *V. tasmaniensis* J0-13, J5-13, and J5-9 were deposited in GenBank (BioProject accession no. PRJNA520819, nucleotide accession nos. SEOP000000000 (J0-13), SESM000000000 (J5-13), and SESL000000000 (J5-9) (74). The genome sequence of LMG20012T was given the BioProject accession no. PRJNA542263, nucleotide accession no. VARQ000000000 (75). Other *Vibrio* genomes presented in this study were previously deposited under the following accession numbers: J2-8 (PRJEB5890), J2-9 (PRJEB5876), J5-4 (PRJEB5877), LGP8 (PRJEB5884), J5-20 (PRJEB5882), and LGP32 (PRJEA32815).

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1. A. F. Takemura, D. M. Chien, M. F. Polz, Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Front. Microbiol.* **5**, 38 (2014).
2. N. Kremer *et al.*, Initial symbiont contact orchestrates host-organ-wide transcriptional changes that prime tissue colonization. *Cell Host Microbe* **14**, 183–194 (2013).
3. M. McFall-Ngai, Divining the essence of symbiosis: Insights from the squid-vibrio model. *PLoS Biol.* **12**, e1001783 (2014).
4. S. Chakraborty, G. B. Nair, S. Shinoda, Pathogenic vibrios in the natural aquatic environment. *Rev. Environ. Health* **12**, 63–80 (1997).

5. J. Dubert, J. L. Barja, J. L. Romalde, New insights into pathogenic vibrios affecting bivalves in hatcheries: Present and future prospects. *Front. Microbiol.* **8**, 762 (2017).
6. F. Le Roux, M. Blokesch, Eco-evolutionary dynamics linked to horizontal gene transfer in vibrios. *Annu. Rev. Microbiol.* **72**, 89–110 (2018).
7. F. L. Thompson, T. Iida, J. Swings, Biodiversity of vibrios. *Microbiol. Mol. Biol. Rev.* **68**, 403–431 (2004).
8. F. Le Roux, K. M. Wegner, M. F. Polz, Oysters and vibrios as a model for disease dynamics in wild animals. *Trends Microbiol.* **24**, 568–580 (2016).

9. P. Schmitt *et al.*, The antimicrobial defense of the pacific oyster, *Crassostrea gigas*. How diversity may compensate for scarcity in the regulation of resident/pathogenic microflora. *Front. Microbiol.* **3**, 160 (2012).
10. J. de Lorgeril *et al.*, Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nat. Commun.* **9**, 4215 (2018).
11. M. Bruto *et al.*, *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME J.* **11**, 1043–1052 (2017).
12. M. Bruto *et al.*, Ancestral gene acquisition as the key to virulence potential in environmental *Vibrio* populations. *ISME J.* **12**, 2954–2966 (2018).
13. M. Gay, F. C. Berthe, F. Le Roux, Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. *Dis. Aquat. Organ.* **59**, 49–56 (2004).
14. A. Lemire *et al.*, Populations, not clones, are the unit of *vibrio* pathogenesis in naturally infected oysters. *ISME J.* **9**, 1523–1531 (2015).
15. M. Duperthuy *et al.*, Use of *OmpU* porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 2993–2998 (2011).
16. A. S. Vanhove *et al.*, Copper homeostasis at the host *vibrio* interface: Lessons from intracellular *vibrio* transcriptomics. *Environ. Microbiol.* **18**, 875–888 (2016).
17. A. S. Vanhove *et al.*, Outer membrane vesicles are vehicles for the delivery of *Vibrio tasmaniensis* virulence factors to oyster immune cells. *Environ. Microbiol.* **17**, 1152–1165 (2015).
18. A. J. Westermann, L. Barquist, J. Vogel, Resolving host-pathogen interactions by dual RNA-seq. *PLoS Pathog.* **13**, e1006033 (2017).
19. C. T. Robb, E. A. Dyrnyda, R. D. Gray, A. G. Rossi, V. J. Smith, Invertebrate extracellular phagocyte traps show that chromatin is an ancient defence weapon. *Nat. Commun.* **5**, 4627 (2014).
20. A. C. Poirier *et al.*, Antimicrobial histones and DNA traps in invertebrate immunity: Evidences in *Crassostrea gigas*. *J. Biol. Chem.* **289**, 24821–24831 (2014).
21. M. W. Hornef, M. J. Wick, M. Rhen, S. Normark, Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat. Immunol.* **3**, 1033–1040 (2002).
22. L. E. Reddick, N. M. Alto, Bacteria fighting back: How pathogens target and subvert the host innate immune system. *Mol. Cell* **54**, 321–328 (2014).
23. B. Beutler, Tlr4: Central component of the sole mammalian LPS sensor. *Curr. Opin. Immunol.* **12**, 20–26 (2000).
24. M. Oldenburg *et al.*, TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. *Science* **337**, 1111–1115 (2012).
25. I. C. McDowell *et al.*, Transcriptome of American oysters, *Crassostrea virginica*, in response to bacterial challenge: Insights into potential mechanisms of disease resistance. *PLoS One* **9**, e105097 (2014).
26. R. D. Rosa *et al.*, Big defensins, a diverse family of antimicrobial peptides that follows different patterns of expression in hemocytes of the oyster *Crassostrea gigas*. *PLoS One* **6**, e25594 (2011).
27. Y. S. Bae, M. K. Choi, W. J. Lee, Dual oxidase in mucosal immunity and host-microbe homeostasis. *Trends Immunol.* **31**, 278–287 (2010).
28. C. L. Bevins, N. H. Salzman, Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat. Rev. Microbiol.* **9**, 356–368 (2011).
29. Z. Jia *et al.*, An integrin from oyster *Crassostrea gigas* mediates the phagocytosis toward *Vibrio splendidus* through LPS binding activity. *Dev. Comp. Immunol.* **53**, 253–264 (2015).
30. X. Pang *et al.*, Mosquito C-type lectins maintain gut microbiome homeostasis. *Nat. Microbiol.* **1**, 16023 (2016).
31. X. W. Wang, J. D. Xu, X. F. Zhao, G. R. Vasta, J. X. Wang, A shrimp C-type lectin inhibits proliferation of the hemolytic microbiota by maintaining the expression of antimicrobial peptides. *J. Biol. Chem.* **289**, 11779–11790 (2014).
32. P. Rosenstiel *et al.*, Regulation of DMBT1 via NOD2 and TLR4 in intestinal epithelial cells modulates bacterial recognition and invasion. *J. Immunol.* **178**, 8203–8211 (2007).
33. J. Li, M. M. E. Metruccio, D. J. Evans, S. M. J. Fleiszig, Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen *Pseudomonas aeruginosa*. *PLoS Pathog.* **13**, e1006392 (2017).
34. Z. Lv *et al.*, Comparative study of three C1q domain containing proteins from pacific oyster *Crassostrea gigas*. *Dev. Comp. Immunol.* **78**, 42–51 (2018).
35. A. F. Takemura *et al.*, Natural resource landscapes of a marine bacterium reveal distinct fitness-determining genes across the genome. *Environ. Microbiol.* **19**, 2422–2433 (2017).
36. M. Basler, Type VI secretion system: Secretion by a contractile nanomachine. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **370**, 20150021 (2015).
37. B. T. Ho, T. G. Dong, J. J. Mekalanos, A view to a kill: The bacterial type VI secretion system. *Cell Host Microbe* **15**, 9–21 (2014).
38. A. Joshi *et al.*, Rules of engagement: The type VI secretion system in *Vibrio cholerae*. *Trends Microbiol.* **25**, 267–279 (2017).
39. V. S. Nguyen *et al.*, Towards a complete structural deciphering of Type VI secretion system. *Curr. Opin. Struct. Biol.* **49**, 77–84 (2018).
40. A. B. Russell, S. B. Peterson, J. D. Mougou, Type VI secretion system effectors: Poisons with a purpose. *Nat. Rev. Microbiol.* **12**, 137–148 (2014).
41. X. Wang *et al.*, *Edwardsiella tarda* T6SS component *evpP* is regulated by *esrB* and iron, and plays essential roles in the invasion of fish. *Fish Shellfish Immunol.* **27**, 469–477 (2009).
42. H. Chen *et al.*, The bacterial T6SS effector *EvpP* prevents NLRP3 inflammasome activation by inhibiting the  $Ca^{2+}$ -dependent MAPK-jnk pathway. *Cell Host Microbe* **21**, 47–58 (2017).
43. J. Lennings, T. E. West, S. Schwarz, The *Burkholderia* type VI secretion system 5: Composition, regulation and role in virulence. *Front. Microbiol.* **9**, 3339 (2019).
44. A. T. Ma, J. J. Mekalanos, In vivo actin cross-linking induced by *Vibrio cholerae* type VI secretion system is associated with intestinal inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 4365–4370 (2010).
45. A. T. Ma, S. McAuley, S. Pukatzki, J. J. Mekalanos, Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell Host Microbe* **5**, 234–243 (2009).
46. L. Speare *et al.*, Bacterial symbionts use a type VI secretion system to eliminate competitors in their natural host. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E8528–E8537 (2018).
47. Y. Labreuche *et al.*, Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. *Microbes Infect.* **8**, 2715–2724 (2006).
48. Y. Labreuche *et al.*, *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. *Fish Shellfish Immunol.* **29**, 753–758 (2010).
49. L. Parizehad *et al.*, Ecologically realistic model of infection for exploring the host damage caused by *Vibrio aestuarianus*. *Environ. Microbiol.* **20**, 4343–4355 (2018).
50. S. F. Bailey, N. Rodrigue, R. Kassen, The effect of selection environment on the probability of parallel evolution. *Mol. Biol. Evol.* **32**, 1436–1448 (2015).
51. B. Petton, F. Pernet, R. Robert, P. Boudry, Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. (Translated from English). *Aquacult. Environ. Interact.* **3**, 257–273 (2013).
52. M. Duperthuy *et al.*, The major outer membrane protein *OmpU* of *Vibrio splendidus* contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*. *Environ. Microbiol.* **12**, 951–963 (2010).
53. F. Le Roux, J. Binesse, D. Saulnier, D. Mazel, Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl. Environ. Microbiol.* **73**, 777–784 (2007).
54. F. Le Roux, B. M. Davis, M. K. Waldor, Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. *Nucleic Acids Res.* **39**, 1004–1013 (2011).
55. G. Demarre *et al.*, A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative machineries and their cognate *Escherichia coli* host strains. *Res. Microbiol.* **156**, 245–255 (2005).
56. F. Le Roux *et al.*, Genome sequence of *Vibrio splendidus*: An abundant planktonic marine species with a large genotypic diversity. *Environ. Microbiol.* **11**, 1959–1970 (2009).
57. E. Afgan *et al.*, The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* **46**, W537–W544 (2018).
58. G. Zhang *et al.*, The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* **490**, 49–54 (2012).
59. A. Dobin *et al.*, STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
60. S. Anders, P. T. Pyl, W. Huber, HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).
61. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
62. B. Buchfink, C. Xie, D. H. Huson, Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* **12**, 59–60 (2015).
63. T. Rubio, E. Toulza, C. Chaparro, G. M. Charrière, D. Destoumieux-Garçon, *Crassostrea gigas* response to *Vibrio* strains J2-9, LGP32, J2-8 and LMG20012T, strain:Decipher Family 14 (Pacific oyster). BioProject. <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA515169>. Deposited 15 January 2019.
64. A. M. Bolger, M. Lohse, B. Usadel, Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
65. B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359 (2012).
66. Y. Liao, G. K. Smyth, W. Shi, FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).
67. T. Rubio, X. Luo, G. M. Charrière, D. Destoumieux-Garçon, A. Jacq, *Vibrio crassostreae* strain:J2-9 response to oyster colonization. BioProject. <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA521688>. Deposited 12 February 2019.
68. T. Rubio, X. Luo, G. M. Charrière, D. Destoumieux-Garçon, A. Jacq, *Vibrio tasmaniensis* strain: LGP32 response to oyster colonization. BioProject. <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA521693>. Deposited 11 February 2019.
69. S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
70. A. Conesa *et al.*, Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674–3676 (2005).
71. C. Médigue *et al.*, MicroScope—an integrated resource for community expertise of gene functions and comparative analysis of microbial genomic and metabolic data. *Brief. Bioinform.* **10**, 1093/bib/bbx113 (2017).
72. R. Caspi *et al.*, The MetaCyc database of metabolic pathways and enzymes. *Nucleic Acids Res.* **46**, D633–D639 (2018).
73. D. Vallenet *et al.*, MicroScope—An integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res.* **41**, D636–D647 (2013).
74. M. Bruto, A. Lagorce, E. Toulza, F. Le Roux, D. Destoumieux-Garçon, *Vibrio tasmaniensis*, strains: J0-13, J5-13 and J5-9. BioProject. <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA520819>. Deposited 4 February 2019.
75. M. Bruto, G. M. Charrière, F. Le Roux, D. Destoumieux-Garçon, *Vibrio tasmaniensis* strain: LMG 20012T. BioProject. <https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA542263>. Deposited 5 May 2019.

## REFERENCES

- Abisado RG, Benomar S, Klaus JR, Dandekar AA, Chandler JR (2018) Bacterial Quorum Sensing and Microbial Community Interactions. *MBio* **9**
- Altuvia S, Weinstein-Fischer D, Zhang A, Postow L, Storz G (1997) A Small, Stable RNA Induced by Oxidative Stress: Role as a Pleiotropic Regulator and Antimutator. *Cell* **90**: 43-53
- Ames BN, Tsang TH, Buck M, Christman MF (1983) The leader mRNA of the histidine attenuator region resembles tRNA<sup>His</sup>: possible general regulatory implications. *Proceedings of the National Academy of Sciences of the United States of America* **80**: 5240-5242
- Ancona V, Lee JH, Zhao Y (2016) The RNA-binding protein CsrA plays a central role in positively regulating virulence factors in *Erwinia amylovora*. *Scientific reports* **6**: 37195
- Andersen J, Delihans N, Ikenaka K, Green PJ, Pines O, Ilercil O, Inouye M (1987) The isolation and characterization of RNA coded by the micF gene in *Escherichia coli*. *Nucleic Acids Res* **15**: 2089-2101
- Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M, Molin S (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**: 2240-2246
- Argaman L, Hershberg R, Vogel J, Bejerano G, Wagner EG, Margalit H, Altuvia S (2001) Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Current biology : CB* **11**: 941-950
- Austin B (2010) Vibrios as causal agents of zoonoses. *Veterinary microbiology* **140**: 310-317
- Austin B, Austin DA (2016) Vibrios. In *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*, pp 499-601. Cham: Springer International Publishing
- Azhikina TL, Ignatov DV, Salina EG, Fursov MV, Kaprelyants AS (2015) Role of Small Noncoding RNAs in Bacterial Metabolism. *Biochemistry Biokhimiia* **80**: 1633-1646
- Babitzke P, Romeo T (2007) CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr Opin Microbiol* **10**: 156-163
- Baccigalupi L, Marasco R, Ricca E, De Felice M, Sacco M (1995) Control of ilvIH transcription during amino acid downshift in stringent and relaxed strains of *Escherichia coli*. *FEMS Microbiol Lett* **131**: 95-98
- Baker CS, Morozov I, Suzuki K, Romeo T, Babitzke P (2002) CsrA regulates glycogen biosynthesis by

preventing translation of *glgC* in *Escherichia coli*. *Mol Microbiol* **44**: 1599-1610

Balaban N, Novick RP (1995) Translation of RNAIII, the *Staphylococcus aureus* agr regulatory RNA molecule, can be activated by a 3'-end deletion. *FEMS microbiology letters* **133**: 155-161

Balbontín R, Fiorini F, Figueroa-Bossi N, Casadesús J, Bossi L (2010) Recognition of heptameric seed sequence underlies multi-target regulation by RybB small RNA in *Salmonella enterica*. *Molecular Microbiology* **78**: 380-394

Bardill JP, Hammer BK (2012) Non-coding sRNAs regulate virulence in the bacterial pathogen *Vibrio cholerae*. *RNA Biol* **9**: 392-401

Bardill JP, Zhao X, Hammer BK (2011) The *Vibrio cholerae* quorum sensing response is mediated by Hfq-dependent sRNA/mRNA base pairing interactions. *Mol Microbiol* **80**: 1381-1394

Barrick JE, Corbino KA, Winkler WC, Nahvi A, Mandal M, Collins J, Lee M, Roth A, Sudarsan N, Jona I, Wickiser JK, Breaker RR (2004) New RNA motifs suggest an expanded scope for riboswitches in bacterial genetic control. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 6421-6426

Barrick JE, Sudarsan N, Weinberg Z, Ruzzo WL, Breaker RR (2005) 6S RNA is a widespread regulator of eubacterial RNA polymerase that resembles an open promoter. *RNA* **11**: 774-784

Bassler BL, Wright M, Silverman MR (1994) Sequence and function of LuxO, a negative regulator of luminescence in *Vibrio harveyi*. *Mol Microbiol* **12**: 403-412

Beisel CL, Storz G (2011) The base-pairing RNA spot 42 participates in a multioutput feedforward loop to help enact catabolite repression in *Escherichia coli*. *Mol Cell* **41**: 286-297

Benito Y, Kolb FA, Romby P, Lina G, Etienne J, Vandenesch F (2000) Probing the structure of RNAIII, the *Staphylococcus aureus* agr regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *RNA* **6**: 668-679

Bernhart SH, Hofacker IL, Will S, Gruber AR, Stadler PF (2008) RNAalifold: improved consensus structure prediction for RNA alignments. *BMC Bioinformatics* **9**: 474

Bilusic I, Popitsch N, Rescheneder P, Schroeder R, Lybecker M (2014) Revisiting the coding potential of the *E. coli* genome through Hfq co-immunoprecipitation. *RNA Biology* **11**: 641-654

Binesse J, Delsert C, Saulnier D, Champomier-Verges MC, Zagorec M, Munier-Lehmann H, Mazel D, Le Roux F (2008) Metalloprotease vsm is the major determinant of toxicity for extracellular products of *Vibrio splendidus*. *Applied and environmental microbiology* **74**: 7108-7117

Bohn C, Rigoulay C, Bouloc P (2007) No detectable effect of RNA-binding protein Hfq absence in *Staphylococcus aureus*. *BMC Microbiol* **7**: 10

Bohn C, Rigoulay C, Chabelskaya S, Sharma CM, Marchais A, Skorski P, Borezee-Durant E, Barbet R, Jacquet E, Jacq A, Gautheret D, Felden B, Vogel J, Bouloc P (2010) Experimental discovery of small RNAs in *Staphylococcus aureus* reveals a riboregulator of central metabolism. *Nucleic Acids Res* **38**: 6620-6636

Boos W, Shuman H (1998) Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microbiol Mol Biol Rev* **62**: 204-229

Bossi L, Schwartz A, Guillemardet B, Boudvillain M, Figueroa-Bossi N (2012) A role for Rho-dependent polarity in gene regulation by a noncoding small RNA. *Genes Dev* **26**: 1864-1873

Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J (2008) Small RNA binding to 5' mRNA coding region inhibits translational initiation. *Mol Cell* **32**: 827-837

Bradley ES, Bodi K, Ismail AM, Camilli A (2011) A genome-wide approach to discovery of small RNAs involved in regulation of virulence in *Vibrio cholerae*. *PLoS Pathog* **7**: e1002126

Brantl S, Bruckner R (2014) Small regulatory RNAs from low-GC Gram-positive bacteria. *RNA Biol* **11**: 443-456

Breaker RR (2018) Riboswitches and Translation Control. *Cold Spring Harb Perspect Biol* **10**

Brinsmade SR (2017) CodY, a master integrator of metabolism and virulence in Gram-positive bacteria. *Curr Genet* **63**: 417-425

Bronesky D, Wu Z, Marzi S, Walter P, Geissmann T, Moreau K, Vandenesch F, Caldelari I, Romby P (2016) *Staphylococcus aureus* RNAIII and Its Regulon Link Quorum Sensing, Stress Responses, Metabolic Adaptation, and Regulation of Virulence Gene Expression. *Annu Rev Microbiol* **70**: 299-316

Browning DF, Busby SJ (2004) The regulation of bacterial transcription initiation. *Nature reviews Microbiology* **2**: 57-65

Brownlee GG (1971) Sequence of 6S RNA of *E. coli*. *Nature: New biology* **229**: 147-149

Bruckner R, Titgemeyer F (2002) Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. *FEMS Microbiol Lett* **209**: 141-148

Bruto M, James A, Petton B, Labreuche Y, Chenivesse S, Alunno-Bruscia M, Polz MF, Le Roux F

(2017) *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *The ISME journal* **11**: 1043-1052

Bruto M, Labreuche Y, James A, Piel D, Chenivesse S, Petton B, Polz MF, Le Roux F (2018) Ancestral gene acquisition as the key to virulence potential in environmental *Vibrio* populations. *The ISME journal* **12**: 2954-2966

Burenina OY, Hoch PG, Damm K, Salas M, Zatsopin TS, Lechner M, Oretskaya TS, Kubareva EA, Hartmann RK (2014) Mechanistic comparison of *Bacillus subtilis* 6S-1 and 6S-2 RNAs-commonalities and differences. *RNA* **20**: 348-359

Caron MP, Lafontaine DA, Masse E (2010) Small RNA-mediated regulation at the level of transcript stability. *RNA Biol* **7**: 140-144

Carroll PA, Tashima KT, Rogers MB, DiRita VJ, Calderwood SB (1997) Phase variation in tcpH modulates expression of the ToxR regulon in *Vibrio cholerae*. *Mol Microbiol* **25**: 1099-1111

Carroll RK, Weiss A, Broach WH, Wiemels RE, Mogen AB, Rice KC, Shaw LN (2016) Genome-wide Annotation, Identification, and Global Transcriptomic Analysis of Regulatory or Small RNA Gene Expression in *Staphylococcus aureus*. *MBio* **7**: e01990-01915

Carter PW, Bartkus JM, Calvo JM (1986) Transcription attenuation in *Salmonella typhimurium*: the significance of rare leucine codons in the *leu* leader. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 8127-8131

Cavanagh AT, Sperger JM, Wassarman KM (2012) Regulation of 6S RNA by pRNA synthesis is required for efficient recovery from stationary phase in *E. coli* and *B. subtilis*. *Nucleic Acids Res* **40**: 2234-2246

Cavanagh AT, Wassarman KM (2013) 6S-1 RNA function leads to a delay in sporulation in *Bacillus subtilis*. *J Bacteriol* **195**: 2079-2086

Cavanagh AT, Wassarman KM (2014) 6S RNA, a global regulator of transcription in *Escherichia coli*, *Bacillus subtilis*, and beyond. *Annu Rev Microbiol* **68**: 45-60

Chakraborty S, Nair GB, Shinoda S (1997) Pathogenic vibrios in the natural aquatic environment. *Reviews on environmental health* **12**: 63-80

Chang C, Jin X, Chaoqun H (2009) Phenotypic and genetic differences between opaque and translucent colonies of *Vibrio alginolyticus*. *Biofouling* **25**: 525-531

Chang H, Replogle JM, Vather N, Tsao-Wu M, Mistry R, Liu JM (2015) A cis-regulatory antisense

RNA represses translation in *Vibrio cholerae* through extensive complementarity and proximity to the target locus. *RNA Biol* **12**: 136-148

Chao Y, Papenfort K, Reinhardt R, Sharma CM, Vogel J (2012) An atlas of Hfq-bound transcripts reveals 3' UTRs as a genomic reservoir of regulatory small RNAs. *EMBO J* **31**: 4005-4019

Chao Y, Vogel J (2016) A 3' UTR-Derived Small RNA Provides the Regulatory Noncoding Arm of the Inner Membrane Stress Response. *Mol Cell* **61**: 352-363

Chareyre S, Mandin P (2018) Bacterial Iron Homeostasis Regulation by sRNAs. *Microbiol Spectr* **6**

Chen J, Gottesman S (2017) Hfq links translation repression to stress-induced mutagenesis in *E. coli*. *Genes Dev* **31**: 1382-1395

Chen J, Wassarman KM, Feng S, Leon K, Feklistov A, Winkelman JT, Li Z, Walz T, Campbell EA, Darst SA (2017a) 6S RNA Mimics B-Form DNA to Regulate *Escherichia coli* RNA Polymerase. *Mol Cell* **68**: 388-397 e386

Chen M, Zhao Z, Yang J, Peng K, Baker MA, Bai F, Lo CJ (2017b) Length-dependent flagellar growth of *Vibrio alginolyticus* revealed by real time fluorescent imaging. *Elife* **6**: e22140

Chen Q, Crosa JH (1996) Antisense RNA, fur, iron, and the regulation of iron transport genes in *Vibrio anguillarum*. *J Biol Chem* **271**: 18885-18891

Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549

Childers BM, Klose KE (2007) Regulation of virulence in *Vibrio cholerae*: the ToxR regulon. *Future microbiology* **2**: 335-344

Christiansen JK, Nielsen JS, Ebersbach T, Valentin-Hansen P, Sogaard-Andersen L, Kallipolitis BH (2006) Identification of small Hfq-binding RNAs in *Listeria monocytogenes*. *RNA* **12**: 1383-1396

Citil BE, Derin S, Sankur F, Sahan M, Citil MU (2015) *Vibrio alginolyticus* Associated Chronic Myringitis Acquired in Mediterranean Waters of Turkey. *Case reports in infectious diseases* **2015**: 187212

Coornaert A, Chiaruttini C, Springer M, Guillier M (2013) Post-transcriptional control of the *Escherichia coli* PhoQ-PhoP two-component system by multiple sRNAs involves a novel pairing region of GcvB. *PLoS Genet* **9**: e1003156

Coornaert A, Lu A, Mandin P, Springer M, Gottesman S, Guillier M (2010) MicA sRNA links the PhoP

regulon to cell envelope stress. *Mol Microbiol* **76**: 467-479

Crosby HA, Schlievert PM, Merriman JA, King JM, Salgado-Pabon W, Horswill AR (2016) The *Staphylococcus aureus* Global Regulator MgrA Modulates Clumping and Virulence by Controlling Surface Protein Expression. *PLoS Pathog* **12**: e1005604

Dambach M, Irnov I, Winkler WC (2013) Association of RNAs with *Bacillus subtilis* Hfq. *PLoS One* **8**: e55156

Davis BM, Quinones M, Pratt J, Ding Y, Waldor MK (2005) Characterization of the small untranslated RNA RyhB and its regulon in *Vibrio cholerae*. *J Bacteriol* **187**: 4005-4014

Davis BM, Waldor MK (2007) RNase E-dependent processing stabilizes MicX, a *Vibrio cholerae* sRNA. *Mol Microbiol* **65**: 373-385

De Fernandez MF, Eoyang L, August J (1968) Factor fraction required for the synthesis of bacteriophage Q $\beta$ -RNA. *Nature* **219**: 588

De Lay N, Gottesman S (2012) A complex network of small non-coding RNAs regulate motility in *Escherichia coli*. *Mol Microbiol* **86**: 524-538

De Lorgeril J, Escoubas JM, Loubiere V, Pernet F, Le Gall P, Vergnes A, Aujoulat F, Jeannot JL, Jumas-Bilak E, Got P, Gueguen Y, Destoumieux-Garzon D, Bachere E (2018a) Inefficient immune response is associated with microbial permissiveness in juvenile oysters affected by mass mortalities on field. *Fish & shellfish immunology* **77**: 156-163

De Lorgeril J, Lucasson A, Petton B, Toulza E, Montagnani C, Clerissi C, Vidal-Dupiol J, Chaparro C, Galinier R, Escoubas JM, Haffner P, Degremont L, Charriere GM, Lafont M, Delort A, Vergnes A, Chiarello M, Faury N, Rubio T, Leroy MA, Perignon A, Regler D, Morga B, Alunno-Bruscia M, Boudry P, Le Roux F, Destoumieux-Garzon D, Gueguen Y, Mitta G (2018b) Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nat Commun* **9**: 4215

De Mets F, Van Melderen L, Gottesman S (2019) Regulation of acetate metabolism and coordination with the TCA cycle via a processed small RNA. *Proceedings of the National Academy of Sciences of the United States of America* **116**: 1043-1052

Deane EE, Li J, Woo NY (2004) Modulated heat shock protein expression during pathogenic *Vibrio alginolyticus* stress of sea bream. *Dis Aquat Organ* **62**: 205-215

Deng Y, Chen C, Zhao Z, Huang X, Yang Y, Ding X (2016a) Complete Genome Sequence of *Vibrio alginolyticus* ZJ-T. *Genome Announc* **4**

- Deng Y, Chen C, Zhao Z, Zhao J, Jacq A, Huang X, Yang Y (2016b) The RNA Chaperone Hfq Is Involved in Colony Morphology, Nutrient Utilization and Oxidative and Envelope Stress Response in *Vibrio alginolyticus*. *PLoS One* **11**: e0163689
- Deng Y, Su Y, Liu S, Guo Z, Cheng C, Ma H, Wu J, Feng J, Chen C (2018) Identification of a Novel Small RNA sryg23535 in *Vibrio alginolyticus* ZJ-T and Its Characterization With Phenotype MicroArray Technology. *Front Microbiol* **9**: 2394
- Desnoyers G, Morissette A, Prevost K, Masse E (2009) Small RNA-induced differential degradation of the polycistronic mRNA iscRSUA. *EMBO J* **28**: 1551-1561
- Dimastrogiovanni D, Frohlich KS, Bandyra KJ, Bruce HA, Hohensee S, Vogel J, Luisi BF (2014) Recognition of the small regulatory RNA RydC by the bacterial Hfq protein. *Elife* **3**: e05375
- Dubey AK, Baker CS, Suzuki K, Jones AD, Pandit P, Romeo T, Babitzke P (2003) CsrA regulates translation of the *Escherichia coli* carbon starvation gene, *cstA*, by blocking ribosome access to the *cstA* transcript. *J Bacteriol* **185**: 4450-4460
- Duperthuy M, Binesse J, Le Roux F, Romestand B, Caro A, Got P, Givaudan A, Mazel D, Bachere E, Destoumieux-Garzon D (2010) The major outer membrane protein OmpU of *Vibrio splendidus* contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*. *Environmental microbiology* **12**: 951-963
- Duperthuy M, Schmitt P, Garzon E, Caro A, Rosa RD, Le Roux F, Lautredou-Audouy N, Got P, Romestand B, de Lorgeril J, Kieffer-Jaquinod S, Bachere E, Destoumieux-Garzon D (2011) Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 2993-2998
- Durica-Mitic S, Gopel Y, Gorke B (2018) Carbohydrate Utilization in Bacteria: Making the Most Out of Sugars with the Help of Small Regulatory RNAs. *Microbiol Spectr* **6**
- Dutta T, Srivastava S (2018) Small RNA-mediated regulation in bacteria: A growing palette of diverse mechanisms. *Gene* **656**: 60-72
- Ellis MJ, Trussler RS, Haniford DB (2015) Hfq binds directly to the ribosome-binding site of IS 10 transposase mRNA to inhibit translation. *Molecular microbiology* **96**: 633-650
- Faubladier M, Cam K, Bouche JP (1990) *Escherichia coli* cell division inhibitor DicF-RNA of the *dicB* operon. Evidence for its generation in vivo by transcription termination and by RNase III and RNase E-dependent processing. *J Mol Biol* **212**: 461-471

Felden B, Vandenesch F, Bouloc P, Romby P (2011) The *Staphylococcus aureus* RNome and its commitment to virulence. *PLoS Pathog* **7**: e1002006

Feng L, Rutherford ST, Papenfort K, Bagert JD, van Kessel JC, Tirrell DA, Wingreen NS, Bassler BL (2015) A *qrr* noncoding RNA deploys four different regulatory mechanisms to optimize quorum-sensing dynamics. *Cell* **160**: 228-240

Figuroa-Bossi N, Schwartz A, Guillemardet B, D'Heygere F, Bossi L, Boudvillain M (2014) RNA remodeling by bacterial global regulator CsrA promotes Rho-dependent transcription termination. *Genes Dev* **28**: 1239-1251

Fozo EM, Makarova KS, Shabalina SA, Yutin N, Koonin EV, Storz G (2010) Abundance of type I toxin-antitoxin systems in bacteria: searches for new candidates and discovery of novel families. *Nucleic Acids Res* **38**: 3743-3759

Friden P, Newman T, Freundlich M (1982) Nucleotide sequence of the *ilvB* promoter-regulatory region: a biosynthetic operon controlled by attenuation and cyclic AMP. *Proc Natl Acad Sci U S A* **79**: 6156-6160

Frohlich KS, Gottesman S (2018) Small Regulatory RNAs in the Enterobacterial Response to Envelope Damage and Oxidative Stress. *Microbiol Spectr* **6**

Frohlich KS, Papenfort K, Fekete A, Vogel J (2013) A small RNA activates CFA synthase by isoform-specific mRNA stabilization. *EMBO J* **32**: 2963-2979

Frohlich KS, Vogel J (2009) Activation of gene expression by small RNA. *Curr Opin Microbiol* **12**: 674-682

Garcia K, Yanez C, Plaza N, Pena F, Sepulveda P, Perez-Reytor D, Espejo RT (2017) Gene expression of *Vibrio parahaemolyticus* growing in laboratory isolation conditions compared to those common in its natural ocean environment. *BMC Microbiol* **17**: 118

Gay M, Berthe FC, Le Roux F (2004a) Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. *Dis Aquat Organ* **59**: 49-56

Gay M, Renault T, Pons AM, Le Roux F (2004b) Two *vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. *Dis Aquat Organ* **62**: 65-74

Geisinger E, Adhikari RP, Jin R, Ross HF, Novick RP (2006) Inhibition of rot translation by RNAIII, a key feature of *agr* function. *Mol Microbiol* **61**: 1038-1048

Geissmann T, Chevalier C, Cros MJ, Boisset S, Fechter P, Noirot C, Schrenzel J, Francois P, Vandenesch

F, Gaspin C, Romby P (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. *Nucleic Acids Res* **37**: 7239-7257

Georg J, Hess WR (2011) *cis*-antisense RNA, another level of gene regulation in bacteria. *Microbiol Mol Biol Rev* **75**: 286-300

Gerdes K, Gulyaev AP, Franch T, Pedersen K, Mikkelsen ND (1997) Antisense RNA-regulated programmed cell death. *Annu Rev Genet* **31**: 1-31

Gerdes K, Rasmussen PB, Molin S (1986) Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 3116-3120

Giangrossi M, Prosseda G, Tran CN, Brandi A, Colonna B, Falconi M (2010) A novel antisense RNA regulates at transcriptional level the virulence gene *icsA* of *Shigella flexneri*. *Nucleic Acids Res* **38**: 3362-3375

Gibson DG (2011) Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol* **498**: 349-361

Gildehaus N, Neusser T, Wurm R, Wagner R (2007) Studies on the function of the riboregulator 6S RNA from *E. coli*: RNA polymerase binding, inhibition of in vitro transcription and synthesis of RNA-directed de novo transcripts. *Nucleic Acids Res* **35**: 1885-1896

Gimpel M, Brantl S (2017) Dual-function small regulatory RNAs in bacteria. *Mol Microbiol* **103**: 387-397

Gimpel M, Heidrich N, Mäder U, Krügel H, Brantl S (2010) A dual-function sRNA from *B. subtilis*: SR1 acts as a peptide encoding mRNA on the gapA operon. *Molecular microbiology* **76**: 990-1009

Gollnick P, Babitzke P (2002) Transcription attenuation. *Biochimica et biophysica acta* **1577**: 240-250

Gopel Y, Papenfort K, Reichenbach B, Vogel J, Gorke B (2013) Targeted decay of a regulatory small RNA by an adaptor protein for RNase E and counteraction by an anti-adaptor RNA. *Genes Dev* **27**: 552-564

Gottesman S, Storz G (2011) Bacterial small RNA regulators: versatile roles and rapidly evolving variations. *Cold Spring Harb Perspect Biol* **3**

Gualerzi CO, Brandi L, Caserta E, Garofalo C, Lammi M, La Teana A, Petrelli D, Spurio R, Tomsic J, Pon CL (2001) Initiation factors in the early events of mRNA translation in bacteria. *Cold Spring Harbor symposia on quantitative biology* **66**: 363-376

Guedon E, Serror P, Ehrlich SD, Renault P, Delorme C (2001) Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. *Mol Microbiol* **40**: 1227-1239

Guillier M, Gottesman S (2008) The 5' end of two redundant sRNAs is involved in the regulation of multiple targets, including their own regulator. *Nucleic Acids Res* **36**: 6781-6794

Guo MS, Updegrave TB, Gogol EB, Shabalina SA, Gross CA, Storz G (2014a) MicL, a new sigmaE-dependent sRNA, combats envelope stress by repressing synthesis of Lpp, the major outer membrane lipoprotein. *Genes Dev* **28**: 1620-1634

Guo MS, Updegrave TB, Gogol EB, Shabalina SA, Gross CA, Storz G (2014b) MicL, a new  $\sigma^E$ -dependent sRNA, combats envelope stress by repressing synthesis of Lpp, the major outer membrane lipoprotein. *Genes & development* **28**: 1620-1634

Gupta RK, Alba J, Xiong YQ, Bayer AS, Lee CY (2013) MgrA activates expression of capsule genes, but not the alpha-toxin gene in experimental *Staphylococcus aureus* endocarditis. *J Infect Dis* **208**: 1841-1848

Gupta RK, Luong TT, Lee CY (2015) RNAIII of the *Staphylococcus aureus* agr system activates global regulator MgrA by stabilizing mRNA. *Proceedings of the National Academy of Sciences of the United States of America* **112**: 14036-14041

Gusarov, II, Kreneva RA, Podcharniaev DA, Iomantas Iu V, Abalakina EG, Stoinova NV, Perumov DA, Kozlov Iu I (1997) Riboflavin biosynthetic genes in *Bacillus amyloliquefaciens*: primary structure, organization and regulation of activity. *Molekuliarnaia biologii* **31**: 446-453

Hammer BK, Bassler BL (2007) Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 11145-11149

Hansen GA, Ahmad R, Hjerde E, Fenton CG, Willassen NP, Haugen P (2012) Expression profiling reveals Spot 42 small RNA as a key regulator in the central metabolism of *Aliivibrio salmonicida*. *BMC Genomics* **13**: 37

Hauser CA, Hatfield GW (1983) Nucleotide sequence of the ilvB multivalent attenuator region of *Escherichia coli* K12. *Nucleic Acids Res* **11**: 127-139

Hauser CA, Hatfield GW (1984) Attenuation of the ilvB operon by amino acids reflecting substrates or products of the ilvB gene product. *Proc Natl Acad Sci U S A* **81**: 76-79

Hazen TH, Pan L, Gu JD, Sobecky PA (2010) The contribution of mobile genetic elements to the evolution and ecology of *Vibrios*. *FEMS Microbiol Ecol* **74**: 485-499

Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM (2000) DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**: 477-483

Heidrich N, Chinali A, Gerth U, Brantl S (2006) The small untranslated RNA SR1 from the *Bacillus subtilis* genome is involved in the regulation of arginine catabolism. *Molecular microbiology* **62**: 520-536

Heidrich N, Moll I, Brantl S (2007) In vitro analysis of the interaction between the small RNA SR1 and its primary target *ahrC* mRNA. *Nucleic acids research* **35**: 4331-4346

Heilmann B, Hakkila K, Georg J, Tyystjarvi T, Hess WR, Axmann IM, Dienst D (2017) 6S RNA plays a role in recovery from nitrogen depletion in *Synechocystis sp.* PCC 6803. *BMC Microbiol* **17**: 229

Herzog R, Papenfort K (2018a) Chapter Thirteen-Transcriptomic Approaches for Studying Quorum Sensing in *Vibrio cholerae*. In *Methods in Enzymology*, Carpousis AJ (ed), Vol. 612, pp 303-342. Academic Press

Herzog R, Papenfort K (2018b) Transcriptomic Approaches for Studying Quorum Sensing in *Vibrio cholerae*. *Methods Enzymol* **612**: 303-342

Higgins DA, Pomianek ME, Kraml CM, Taylor RK, Semmelhack MF, Bassler BL (2007) The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* **450**: 883-886

Higgins DE, Nazareno E, DiRita VJ (1992) The virulence gene activator ToxT from *Vibrio cholerae* is a member of the AraC family of transcriptional activators. *J Bacteriol* **174**: 6974-6980

Hindley J (1967a) Fractionation of <sup>32</sup>P-labelled ribonucleic acids on polyacrylamide gels and their characterization by fingerprinting. *J Mol Biol* **30**: 125-136

Hindley JT (1967b) Fractionation of <sup>32</sup>P-labelled ribonucleic acids on polyacrylamide gels and their characterization by fingerprinting. *Journal of molecular biology* **30**: 125-136

Hoekzema M, Romilly C, Holmqvist E, Wagner EGH (2019) Hfq-dependent mRNA unfolding promotes sRNA-based inhibition of translation. *EMBO J* **38**

Holmqvist E, Reimegard J, Sterk M, Grantcharova N, Romling U, Wagner EG (2010) Two antisense

RNAs target the transcriptional regulator CsgD to inhibit curli synthesis. *EMBO J* **29**: 1840-1850

Horstmann N, Orans J, Valentin-Hansen P, Shelburne SA, 3rd, Brennan RG (2012) Structural mechanism of *Staphylococcus aureus* Hfq binding to an RNA A-tract. *Nucleic Acids Res* **40**: 11023-11035

Huang HY, Chang HY, Chou CH, Tseng CP, Ho SY, Yang CD, Ju YW, Huang HD (2009) sRNAMap: genomic maps for small non-coding RNAs, their regulators and their targets in microbial genomes. *Nucleic Acids Res* **37**: D150-154

Ikemura T, Dahlberg JE (1973) Small ribonucleic acids of *Escherichia coli*. I. Characterization by polyacrylamide gel electrophoresis and fingerprint analysis. *J Biol Chem* **248**: 5024-5032

Ingavale S, van Wamel W, Luong TT, Lee CY, Cheung AL (2005) Rat/MgrA, a regulator of autolysis, is a regulator of virulence genes in *Staphylococcus aureus*. *Infect Immun* **73**: 1423-1431

Itoh T, Tomizawa J (1980) Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. *Proceedings of the National Academy of Sciences of the United States of America* **77**: 2450-2454

Jacobs Slifka KM, Newton AE, Mahon BE (2017) *Vibrio alginolyticus* infections in the USA, 1988-2012. *Epidemiology and Infection* **145**: 1491-1499

Jahn N, Brantl S (2013) One antitoxin-two functions: SR4 controls toxin mRNA decay and translation. *Nucleic Acids Res* **41**: 9870-9880

Jain SK, Gurevitz M, Apirion D (1982) A small RNA that complements mutants in the RNA processing enzyme ribonuclease P. *Journal of Molecular Biology* **162**: 515-533

Johansson J, Mandin P, Renzoni A, Chiaruttini C, Springer M, Cossart P (2002) An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* **110**: 551-561

Jousselin A, Metzinger L, Felden B (2009) On the facultative requirement of the bacterial RNA chaperone, Hfq. *Trends Microbiol* **17**: 399-405

Kaiser JC, King AN, Grigg JC, Sheldon JR, Edgell DR, Murphy MEP, Brinsmade SR, Heinrichs DE (2018) Repression of branched-chain amino acid synthesis in *Staphylococcus aureus* is mediated by isoleucine via CodY, and by a leucine-rich attenuator peptide. *PLoS Genet* **14**: e1007159

Kaito C, Saito Y, Ikuo M, Omae Y, Mao H, Nagano G, Fujiyuki T, Numata S, Han X, Obata K, Hasegawa S, Yamaguchi H, Inokuchi K, Ito T, Hiramatsu K, Sekimizu K (2013) Mobile genetic element SCCmec-encoded psm-mec RNA suppresses translation of agrA and attenuates MRSA virulence. *PLoS*

*Pathog* **9**: e1003269

Kaito C, Saito Y, Nagano G, Ikuo M, Omae Y, Hanada Y, Han X, Kuwahara-Arai K, Hishinuma T, Baba T, Ito T, Hiramatsu K, Sekimizu K (2011) Transcription and translation products of the cytolysin gene *psm-mec* on the mobile genetic element SCCmec regulate *Staphylococcus aureus* virulence. *PLoS Pathog* **7**: e1001267

Kavita K, de Mets F, Gottesman S (2018) New aspects of RNA-based regulation by Hfq and its partner sRNAs. *Curr Opin Microbiol* **42**: 53-61

Kawano M, Aravind L, Storz G (2007) An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Mol Microbiol* **64**: 738-754

Kawano M, Reynolds AA, Miranda-Rios J, Storz G (2005) Detection of 5'- and 3'-UTR-derived small RNAs and cis-encoded antisense RNAs in *Escherichia coli*. *Nucleic Acids Res* **33**: 1040-1050

Kim S, Reyes D, Beaume M, Francois P, Cheung A (2014) Contribution of *teg49* small RNA in the 5' upstream transcriptional region of *sarA* to virulence in *Staphylococcus aureus*. *Infection and immunity* **82**: 4369-4379

Kovacikova G, Lin W, Skorupski K (2004) *Vibrio cholerae* AphA uses a novel mechanism for virulence gene activation that involves interaction with the LysR-type regulator AphB at the *tcpPH* promoter. *Mol Microbiol* **53**: 129-142

Krasteva PV, Fong JC, Shikuma NJ, Beyhan S, Navarro MV, Yildiz FH, Sondermann H (2010) *Vibrio cholerae* VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. *Science* **327**: 866-868

Kreuzer KD, Henkin TM (2018) The T-Box Riboswitch: tRNA as an Effector to Modulate Gene Regulation. *Microbiol Spectr* **6**

Krukonis ES, Yu RR, Dirita VJ (2000) The *Vibrio cholerae* ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. *Mol Microbiol* **38**: 67-84

Lacatena RM, Cesareni G (1981) Base pairing of RNA I with its complementary sequence in the primer precursor inhibits ColE1 replication. *Nature* **294**: 623-626

Lakhal F, Bury-Mone S, Nomane Y, Le Goic N, Paillard C, Jacq A (2008) Dj1A, a membrane-anchored DnaJ-like protein, is required for cytotoxicity of clam pathogen *Vibrio tapetis* to hemocytes. *Appl Environ Microbiol* **74**: 5750-5758

Lalaouna D, Eyraud A, Devinck A, Prevost K, Masse E (2019) GcvB small RNA uses two distinct seed regions to regulate an extensive targetome. *Mol Microbiol* **111**: 473-486

Lawther RP, Wek RC, Lopes JM, Pereira R, Taillon BE, Hatfield GW (1987) The complete nucleotide sequence of the *ilvGMEDA* operon of *Escherichia coli* K-12. *Nucleic Acids Res* **15**: 2137-2155

Le Roux F, Binesse J, Saulnier D, Mazel D (2007) Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Applied and environmental microbiology* **73**: 777-784

Le Roux F, Wegner KM, Baker-Austin C, Vezzulli L, Osorio CR, Amaro C, Ritchie JM, Defoirdt T, Destoumieux-Garzon D, Blokesch M, Mazel D, Jacq A, Cava F, Gram L, Wendling CC, Strauch E, Kirschner A, Huehn S (2015) The emergence of *Vibrio* pathogens in Europe: ecology, evolution, and pathogenesis (Paris, 11-12th March 2015). *Front Microbiol* **6**: 830

Le Roux F, Wegner KM, Polz MF (2016) Oysters and Vibrios as a Model for Disease Dynamics in Wild Animals. *Trends Microbiol* **24**: 568-580

Le Roux F, Zouine M, Chakroun N, Binesse J, Saulnier D, Bouchier C, Zidane N, Ma L, Rusniok C, Lajus A, Buchrieser C, Medigue C, Polz MF, Mazel D (2009) Genome sequence of *Vibrio splendidus*: an abundant planctonic marine species with a large genotypic diversity. *Environmental microbiology* **11**: 1959-1970

Lee HJ, Gottesman S (2016) sRNA roles in regulating transcriptional regulators: Lrp and SoxS regulation by sRNAs. *Nucleic Acids Res* **44**: 6907-6923

Lee KK (1995) Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus*, Bloch et Schneider. *Microb Pathog* **19**: 39-48

Lemay JF, Desnoyers G, Blouin S, Heppell B, Bastet L, St-Pierre P, Masse E, Lafontaine DA (2011) Comparative study between transcriptionally- and translationally-acting adenine riboswitches reveals key differences in riboswitch regulatory mechanisms. *PLoS Genet* **7**: e1001278

Lemire A, Goudenège D, Versigny T, Petton B, Calteau A, Labreuche Y, Le Roux F (2014) Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *The ISME journal* **9**: 1523

Leng Y, Vakulskas CA, Zere TR, Pickering BS, Watnick PI, Babitzke P, Romeo T (2016) Regulation of CsrB/C sRNA decay by EIIA(Glc) of the phosphoenolpyruvate: carbohydrate phosphotransferase system. *Mol Microbiol* **99**: 627-639

Lenz DH, Miller MB, Zhu J, Kulkarni RV, Bassler BL (2005) CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Mol Microbiol* **58**: 1186-1202

Lenz DH, Mok KC, Lilley BN, Kulkarni RV, Wingreen NS, Bassler BL (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**: 69-82

Levinthal M, Lejeune P, Danchin A (1994) The H-NS protein modulates the activation of the *ilvIH* operon of *Escherichia coli* K12 by Lrp, the leucine regulatory protein. *Mol Gen Genet* **242**: 736-743

Lilley BN, Bassler BL (2000) Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. *Mol Microbiol* **36**: 940-954

Lin W, Kovacicova G, Skorupski K (2007) The quorum sensing regulator HapR downregulates the expression of the virulence gene transcription factor AphA in *Vibrio cholerae* by antagonizing Lrp- and VpsR-mediated activation. *Mol Microbiol* **64**: 953-967

Lintner RE, Mishra PK, Srivastava P, Martinez-Vaz BM, Khodursky AB, Blumenthal RM (2008) Limited functional conservation of a global regulator among related bacterial genera: Lrp in *Escherichia*, *Proteus* and *Vibrio*. *BMC microbiology* **8**: 60

Liu J, Fu K, Wu C, Qin K, Li F, Zhou L (2018a) "In-Group" Communication in Marine *Vibrio*: A Review of N-Acyl Homoserine Lactones-Driven Quorum Sensing. *Frontiers in cellular and infection microbiology* **8**: 139

Liu JM, Camilli A (2010) A broadening world of bacterial small RNAs. *Curr Opin Microbiol* **13**: 18-23

Liu JM, Livny J, Lawrence MS, Kimball MD, Waldor MK, Camilli A (2009) Experimental discovery of sRNAs in *Vibrio cholerae* by direct cloning, 5S/tRNA depletion and parallel sequencing. *Nucleic Acids Res* **37**: e46

Liu MY, Gui G, Wei B, Preston JF, 3rd, Oakford L, Yuksel U, Giedroc DP, Romeo T (1997) The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*. *J Biol Chem* **272**: 17502-17510

Liu W, Rochat T, Toffano-Nioche C, Le Lam TN, Bouloc P, Morvan C (2018b) Assessment of Bona Fide sRNAs in *Staphylococcus aureus*. *Front Microbiol* **9**: 228

Livny J, Fogel MA, Davis BM, Waldor MK (2005) sRNAPredict: an integrative computational approach to identify sRNAs in bacterial genomes. *Nucleic Acids Res* **33**: 4096-4105

Loh E, Dussurget O, Gripenland J, Vaitkevicius K, Tiensuu T, Mandin P, Repoila F, Buchrieser C, Cossart P, Johansson J (2009) A *trans*-acting riboswitch controls expression of the virulence regulator

PrfA in *Listeria monocytogenes*. *Cell* **139**: 770-779

Loh E, Righetti F, Eichner H, Twittenhoff C, Narberhaus F (2018) RNA Thermometers in Bacterial Pathogens. *Microbiol Spectr* **6**

Lopez-Joven C, Rolland JL, Haffner P, Caro A, Roques C, Carre C, Travers MA, Abadie E, Laabir M, Bonnet D, Destoumieux-Garzon D (2018) Oyster Farming, Temperature, and Plankton Influence the Dynamics of Pathogenic Vibrios in the Thau Lagoon. *Front Microbiol* **9**: 2530

Lotz TS, Suess B (2018) Small-Molecule-Binding Riboswitches. *Microbiol Spectr* **6**

Luong TT, Dunman PM, Murphy E, Projan SJ, Lee CY (2006) Transcription Profiling of the mgrA Regulon in *Staphylococcus aureus*. *J Bacteriol* **188**: 1899-1910

Majdalani N, Chen S, Murrow J, St John K, Gottesman S (2001) Regulation of RpoS by a novel small RNA: the characterization of RprA. *Mol Microbiol* **39**: 1382-1394

Mandin P, Gottesman S (2010) Integrating anaerobic/aerobic sensing and the general stress response through the ArcZ small RNA. *EMBO J* **29**: 3094-3107

Mandlik A, Livny J, Robins WP, Ritchie JM, Mekalanos JJ, Waldor MK (2011) RNA-Seq-based monitoring of infection-linked changes in *Vibrio cholerae* gene expression. *Cell Host Microbe* **10**: 165-174

Mangold M, Siller M, Roppenser B, Vlamincx BJ, Penfound TA, Klein R, Novak R, Novick RP, Charpentier E (2004) Synthesis of group A streptococcal virulence factors is controlled by a regulatory RNA molecule. *Mol Microbiol* **53**: 1515-1527

Manna AC, Kim S, Cengher L, Corvaglia A, Leo S, Francois P, Cheung AL (2018a) Small RNA Teg49 is derived from a sarA transcript and regulates virulence genes independent of SarA in *Staphylococcus aureus*. *Infection and immunity* **86**: e00635-00617

Manna AC, Kim S, Cengher L, Corvaglia A, Leo S, Francois P, Cheung AL (2018b) Small RNA teg49 Is Derived from a sarA Transcript and Regulates Virulence Genes Independent of SarA in *Staphylococcus aureus*. *Infect Immun* **86**

Masachis S, Darfeuille F (2018) Type I Toxin-Antitoxin Systems: Regulating Toxin Expression via Shine-Dalgarno Sequence Sequestration and Small RNA Binding. *Microbiol Spectr* **6**

Masse E, Escorcía FE, Gottesman S (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev* **17**: 2374-2383

- Masse E, Gottesman S (2002) A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 4620-4625
- McCown PJ, Corbino KA, Stav S, Sherlock ME, Breaker RR (2017) Riboswitch diversity and distribution. *RNA* **23**: 995-1011
- Melamed S, Peer A, Faigenbaum-Romm R, Gatt YE, Reiss N, Bar A, Altuvia Y, Argaman L, Margalit H (2016) Global Mapping of Small RNA-Target Interactions in Bacteria. *Mol Cell* **63**: 884-897
- Melior H, Li S, Madhugiri R, Stotzel M, Azarderakhsh S, Barth-Weber S, Baumgardt K, Ziebuhr J, Evguenieva-Hackenberg E (2019a) Transcription attenuation-derived small RNA rTrpL regulates tryptophan biosynthesis gene expression in trans. *Nucleic Acids Res*
- Melior H, Li S, Madhugiri R, Stotzel M, Azarderakhsh S, Barth-Weber S, Baumgardt K, Ziebuhr J, Evguenieva-Hackenberg E (2019b) Transcription attenuation-derived small RNA rTrpL regulates tryptophan biosynthesis gene expression in trans. *Nucleic Acids Res* **47**: 6396-6410
- Mellin JR, Cossart P (2015) Unexpected versatility in bacterial riboswitches. *Trends in genetics : TIG* **31**: 150-156
- Mey AR, Butz HA, Payne SM (2015) *Vibrio cholerae* CsrA Regulates ToxR Levels in Response to Amino Acids and Is Essential for Virulence. *MBio* **6**: e01064
- Mey AR, Craig SA, Payne SM (2005) Characterization of *Vibrio cholerae* RyhB: the RyhB regulon and role of *ryhB* in biofilm formation. *Infect Immun* **73**: 5706-5719
- Miotto P, Forti F, Ambrosi A, Pellin D, Veiga DF, Balazsi G, Gennaro ML, Di Serio C, Ghisotti D, Cirillo DM (2012) Genome-wide discovery of small RNAs in *Mycobacterium tuberculosis*. *PLoS One* **7**: e51950
- Mitra P, Ghosh G, Hafeezunnisa M, Sen R (2017) Rho Protein: Roles and Mechanisms. *Annu Rev Microbiol* **71**: 687-709
- Miyakoshi M, Chao Y, Vogel J (2015) Cross talk between ABC transporter mRNAs via a target mRNA-derived sponge of the GcvB small RNA. *EMBO J* **34**: 1478-1492
- Møller T, Franch T, Højrup P, Keene DR, Bächinger HP, Brennan RG, Valentin-Hansen P (2002) Hfq: A Bacterial Sm-like Protein that Mediates RNA-RNA Interaction. *Molecular Cell* **9**: 23-30
- Morfeldt E, Taylor D, von Gabain A, Arvidson S (1995) Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAlII. *EMBO J* **14**: 4569-4577

Morita MT, Tanaka Y, Kodama TS, Kyogoku Y, Yanagi H, Yura T (1999) Translational induction of heat shock transcription factor sigma32: evidence for a built-in RNA thermosensor. *Genes Dev* **13**: 655-665

Muller P, Gimpel M, Wildenhain T, Brantl S (2019) A new role for CsrA: promotion of complex formation between an sRNA and its mRNA target in *Bacillus subtilis*. *RNA Biol*: 1-16

Munn CB, Marchant HK, Moody AJ (2008) Defences against oxidative stress in vibrios associated with corals. *FEMS Microbiol Lett* **281**: 58-63

Mustachio LM, Aksit S, Mistry RH, Scheffler R, Yamada A, Liu JM (2012) The *Vibrio cholerae* mannitol transporter is regulated posttranscriptionally by the MtlS small regulatory RNA. *J Bacteriol* **194**: 598-606

Nargang FE, Subrahmanyam CS, Umbarger HE (1980) Nucleotide sequence of ilvGEDA operon attenuator region of *Escherichia coli*. *Proc Natl Acad Sci U S A* **77**: 1823-1827

Nasfi H, Travers MA, de Lorgeril J, Habib C, Sannie T, Sorieul L, Gerard J, Avarre JC, Haffner P, Tourbiez D, Renault T, Furones D, Roque A, Pruzzo C, Cheslett D, Gdoura R, Vallaey T (2015) A European epidemiological survey of *Vibrio splendidus* clade shows unexplored diversity and massive exchange of virulence factors. *World journal of microbiology & biotechnology* **31**: 461-475

Nealson KH, Hastings JW (1979) Bacterial bioluminescence: its control and ecological significance. *Microbiological reviews* **43**: 496-518

Nelson JW, Atilho RM, Sherlock ME, Stockbridge RB, Breaker RR (2017) Metabolism of Free Guanidine in Bacteria Is Regulated by a Widespread Riboswitch Class. *Mol Cell* **65**: 220-230

Neusser T, Polen T, Geissen R, Wagner R (2010) Depletion of the non-coding regulatory 6S RNA in *E. coli* causes a surprising reduction in the expression of the translation machinery. *BMC Genomics* **11**: 165

Newman EB, Lin R (1995) Leucine-responsive regulatory protein: a global regulator of gene expression in *E. coli*. *Annual review of microbiology* **49**: 747-775

Newton A, Kendall M, Vugia DJ, Henao OL, Mahon BE (2012) Increasing rates of vibriosis in the United States, 1996-2010: review of surveillance data from 2 systems. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **54 Suppl 5**: S391-395

Nguyen AN, Disconzi E, Charriere GM, Destoumieux-Garzon D, Bouloc P, Le Roux F, Jacq A (2018a) *csrB* Gene Duplication Drives the Evolution of Redundant Regulatory Pathways Controlling

Expression of the Major Toxic Secreted Metalloproteases in *Vibrio tasmaniensis* LGP32. *mSphere* **3**

Nguyen VS, Douzi B, Durand E, Roussel A, Cascales E, Cambillau C (2018b) Towards a complete structural deciphering of Type VI secretion system. *Curr Opin Struct Biol* **49**: 77-84

Nitzan M, Rehani R, Margalit H (2017) Integration of Bacterial Small RNAs in Regulatory Networks. *Annual review of biophysics* **46**: 131-148

Otaka H, Ishikawa H, Morita T, Aiba H (2011) PolyU tail of rho-independent terminator of bacterial small RNAs is essential for Hfq action. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 13059-13064

Panja S, Schu DJ, Woodson SA (2013) Conserved arginines on the rim of Hfq catalyze base pair formation and exchange. *Nucleic Acids Res* **41**: 7536-7546

Papenfort K, Bassler BL (2016) Quorum sensing signal-response systems in Gram-negative bacteria. *Nature reviews Microbiology* **14**: 576-588

Papenfort K, Forstner KU, Cong JP, Sharma CM, Bassler BL (2015) Differential RNA-seq of *Vibrio cholerae* identifies the VqmR small RNA as a regulator of biofilm formation. *Proceedings of the National Academy of Sciences of the United States of America* **112**: E766-775

Papenfort K, Silpe JE, Schramma KR, Cong JP, Seyedsayamdost MR, Bassler BL (2017) A *Vibrio cholerae* autoinducer-receptor pair that controls biofilm formation. *Nat Chem Biol* **13**: 551-557

Papenfort K, Vogel J (2010) Regulatory RNA in bacterial pathogens. *Cell Host Microbe* **8**: 116-127

Papenfort K, Vogel J (2011) Sweet business: Spot42 RNA networks with CRP to modulate catabolite repression. *Mol Cell* **41**: 245-246

Park H, McGibbon LC, Potts AH, Yakhnin H, Romeo T, Babitzke P (2017) Translational Repression of the RpoS Antiadapter IraD by CsrA Is Mediated via Translational Coupling to a Short Upstream Open Reading Frame. *MBio* **8**

Patterson-Fortin LM, Vakulskas CA, Yakhnin H, Babitzke P, Romeo T (2013) Dual posttranscriptional regulation via a cofactor-responsive mRNA leader. *J Mol Biol* **425**: 3662-3677

Perez-Cataluna A, Lucena T, Tarazona E, Arahal DR, Macian MC, Pujalte MJ (2016) An MLSA approach for the taxonomic update of the *Splendidus* clade, a lineage containing several fish and shellfish pathogenic *Vibrio spp.* *Syst Appl Microbiol* **39**: 361-369

Perez-Reytor D, Plaza N, Espejo RT, Navarrete P, Bastias R, Garcia K (2016) Role of Non-coding

Regulatory RNA in the Virulence of Human Pathogenic *Vibrios*. *Front Microbiol* **7**: 2160

Pfeiffer V, Papenfort K, Lucchini S, Hinton JC, Vogel J (2009) Coding sequence targeting by MicC RNA reveals bacterial mRNA silencing downstream of translational initiation. *Nature structural & molecular biology* **16**: 840-846

Polayes DA, Rice PW, Garner MM, Dahlberg JE (1988) Cyclic AMP-cyclic AMP receptor protein as a repressor of transcription of the *spf* gene of *Escherichia coli*. *J Bacteriol* **170**: 3110-3114

Potts AH, Guo Y, Ahmer BMM, Romeo T (2019) Role of CsrA in stress responses and metabolism important for *Salmonella* virulence revealed by integrated transcriptomics. *PLoS One* **14**: e0211430

Potts AH, Leng Y, Babitzke P, Romeo T (2018) Examination of Csr regulatory circuitry using epistasis analysis with RNA-seq (Epi-seq) confirms that CsrD affects gene expression via CsrA, CsrB and CsrC. *Scientific reports* **8**: 5373

Raghavan R, Groisman EA, Ochman H (2011) Genome-wide detection of novel regulatory RNAs in *E. coli*. *Genome Res* **21**: 1487-1497

Reen FJ, Almagro-Moreno S, Ussery D, Boyd EF (2006) The genomic code: inferring Vibrionaceae niche specialization. *Nature reviews Microbiology* **4**: 697-704

Reichenbach B, Maes A, Kalamorz F, Hajnsdorf E, Gorke B (2008) The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*. *Nucleic Acids Res* **36**: 2570-2580

Ren J, Sang Y, Qin R, Cui Z, Yao YF (2017) 6S RNA is involved in acid resistance and invasion of epithelial cells in *Salmonella enterica* serovar Typhimurium. *Future microbiology* **12**: 1045-1057

Richard AL, Withey JH, Beyhan S, Yildiz F, DiRita VJ (2010) The *Vibrio cholerae* virulence regulatory cascade controls glucose uptake through activation of TarA, a small regulatory RNA. *Mol Microbiol* **78**: 1171-1181

Richter AS, Backofen R (2012) Accessibility and conservation: general features of bacterial small RNA-mRNA interactions? *RNA Biol* **9**: 954-965

Rivas E, Klein RJ, Jones TA, Eddy SR (2001) Computational identification of noncoding RNAs in *E. coli* by comparative genomics. *Current biology : CB* **11**: 1369-1373

Rochat T, Bohn C, Morvan C, Le Lam TN, Razvi F, Pain A, Toffano-Nioche C, Ponien P, Jacq A, Jacquet E, Fey PD, Gautheret D, Bouloc P (2018) The conserved regulatory RNA RsaE down-regulates the arginine degradation pathway in *Staphylococcus aureus*. *Nucleic Acids Res* **46**: 8803-8816

- Rochat T, Bouloc P, Yang Q, Bossi L, Figueroa-Bossi N (2012) Lack of interchangeability of Hfq-like proteins. *Biochimie* **94**: 1554-1559
- Rochat T, Delumeau O, Figueroa-Bossi N, Noirot P, Bossi L, Dervyn E, Bouloc P (2015) Tracking the Elusive Function of *Bacillus subtilis* Hfq. *PLoS One* **10**: e0124977
- Romeo T (1998) Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Molecular microbiology* **29**: 1321-1330
- Romeo T, Vakulskas CA, Babitzke P (2013) Post-transcriptional regulation on a global scale: form and function of Csr/Rsm systems. *Environmental microbiology* **15**: 313-324
- Rosenberg CE (1962) The place of George M. BEARD in nineteenth-century psychiatry. *Bulletin of the history of medicine* **36**: 245-259
- Rossmann J, Narberhaus F (2016) Exploring the modular nature of riboswitches and RNA thermometers. *Nucleic Acids Res* **44**: 5410-5423
- Rubio T, Oyanedel D, Labreuche Y, Toulza E, Luo X, Bruto M, Chaparro C, Torres M, de Lorgeril J, Haffner P, Vidal-Dupiol J, Lagorce A, Petton B, Mitta G, Jacq A, Le Roux F, Charriere GM, Destoumieux-Garzon D (2019) Species-specific mechanisms of cytotoxicity toward immune cells determine the successful outcome of *Vibrio* infections. *Proceedings of the National Academy of Sciences of the United States of America*: 201905747
- Rutherford ST, van Kessel JC, Shao Y, Bassler BL (2011) AphA and LuxR/HapR reciprocally control quorum sensing in vibrios. *Genes Dev* **25**: 397-408
- Sabharwal D, Song T, Papenfort K, Wai SN (2015) The VrrA sRNA controls a stationary phase survival factor Vrp of *Vibrio cholerae*. *RNA Biol* **12**: 186-196
- Sahagan BG, Dahlberg JE (1979) A small, unstable RNA molecule of *Escherichia coli*: spot 42 RNA. I. Nucleotide sequence analysis. *J Mol Biol* **131**: 573-592
- Salmon K, Yang C, Hatfield G (2006a) Biosynthesis and Regulation of the Branched-Chain Amino Acids<sup>†</sup>. *EcoSal Plus*
- Salmon KA, Yang CR, Hatfield GW (2006b) Biosynthesis and Regulation of the Branched-Chain Amino Acids. *EcoSal Plus* **2**
- Sauer E (2013) Structure and RNA-binding properties of the bacterial LSm protein Hfq. *RNA Biol* **10**: 610-618

- Sauer E, Schmidt S, Weichenrieder O (2012) Small RNA binding to the lateral surface of Hfq hexamers and structural rearrangements upon mRNA target recognition. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 9396-9401
- Sauer E, Weichenrieder O (2011) Structural basis for RNA 3'-end recognition by Hfq. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 13065-13070
- Sawabe T, Kita-Tsukamoto K, Thompson FL (2007) Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. *J Bacteriol* **189**: 7932-7936
- Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AR, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T (2013a) Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. *Front Microbiol* **4**: 414
- Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AR, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T (2013b) Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. *Front Microbiol* **4**: 414
- Schlegel A, Bohm A, Lee SJ, Peist R, Decker K, Boos W (2002) Network regulation of the *Escherichia coli* maltose system. *J Mol Microbiol Biotechnol* **4**: 301-307
- Schmidt M, Zheng P, Delihans N (1995) Secondary structures of *Escherichia coli* antisense micF RNA, the 5'-end of the target ompF mRNA, and the RNA/RNA duplex. *Biochemistry* **34**: 3621-3631
- Schoolnik GK, Yildiz FH (2000) The complete genome sequence of *Vibrio cholerae*: a tale of two chromosomes and of two lifestyles. *Genome Biol* **1**: REVIEWS1016
- Schu DJ, Zhang A, Gottesman S, Storz G (2015) Alternative Hfq-sRNA interaction modes dictate alternative mRNA recognition. *EMBO J* **34**: 2557-2573
- Searles LL, Wessler SR, Calvo JM (1983) Transcription attenuation is the major mechanism by which the leu operon of *Salmonella typhimurium* is controlled. *J Mol Biol* **163**: 377-394
- Sedlyarova N, Shamovsky I, Bharati BK, Epshtein V, Chen J, Gottesman S, Schroeder R, Nudler E (2016) sRNA-Mediated Control of Transcription Termination in *E. coli*. *Cell* **167**: 111-121 e113
- Segarra A, Pepin JF, Arzul I, Morga B, Faury N, Renault T (2010) Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res* **153**: 92-99

Serganov A, Nudler E (2013) A decade of riboswitches. *Cell* **152**: 17-24

Sharma CM, Darfeuille F, Plantinga TH, Vogel J (2007) A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. *Genes Dev* **21**: 2804-2817

Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiss S, Sittka A, Chabas S, Reiche K, Hackermuller J, Reinhardt R, Stadler PF, Vogel J (2010) The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* **464**: 250-255

Sharma CM, Papenfort K, Pernitzsch SR, Mollenkopf HJ, Hinton JC, Vogel J (2011) Pervasive post-transcriptional control of genes involved in amino acid metabolism by the Hfq-dependent GcvB small RNA. *Mol Microbiol* **81**: 1144-1165

Shinhara A, Matsui M, Hiraoka K, Nomura W, Hirano R, Nakahigashi K, Tomita M, Mori H, Kanai A (2011) Deep sequencing reveals as-yet-undiscovered small RNAs in *Escherichia coli*. *BMC Genomics* **12**: 428

Siddiqui R, Alam MM, Naser MN, Otomo Y, Yasmin M, Nessa J, Ahsan CR (2016) Prevalence of *Vibrio alginolyticus* in Sediment Samples of River and Coastal Areas of Bangladesh. **29**: 1-6

Silva IJ, Barahona S, Eyraud A, Lalaouna D, Figueroa-Bossi N, Masse E, Arraiano CM (2019) SraL sRNA interaction regulates the terminator by preventing premature transcription termination of *rho* mRNA. *Proceedings of the National Academy of Sciences of the United States of America* **116**: 3042-3051

Sittka A, Lucchini S, Papenfort K, Sharma CM, Rolle K, Binnewies TT, Hinton JC, Vogel J (2008) Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. *PLoS Genet* **4**: e1000163

Sledjeski DD, Gupta A, Gottesman S (1996) The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in *Escherichia coli*. *The EMBO Journal* **15**: 3993-4000

Someya T, Baba S, Fujimoto M, Kawai G, Kumasaka T, Nakamura K (2012) Crystal structure of Hfq from *Bacillus subtilis* in complex with SELEX-derived RNA aptamer: insight into RNA-binding properties of bacterial Hfq. *Nucleic Acids Res* **40**: 1856-1867

Song T, Mika F, Lindmark B, Liu Z, Schild S, Bishop A, Zhu J, Camilli A, Johansson J, Vogel J, Wai SN (2008) A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. *Mol Microbiol* **70**: 100-111

- Song T, Sabharwal D, Gurung JM, Cheng AT, Sjoström AE, Yildiz FH, Uhlin BE, Wai SN (2014) *Vibrio cholerae* utilizes direct sRNA regulation in expression of a biofilm matrix protein. *PLoS One* **9**: e101280
- Song T, Sabharwal D, Wai SN (2010) VrrA mediates Hfq-dependent regulation of OmpT synthesis in *Vibrio cholerae*. *J Mol Biol* **400**: 682-688
- Sonntag I, Schwarz H, Hirota Y, Henning U (1978) Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. *J Bacteriol* **136**: 280-285
- Stork M, Di Lorenzo M, Welch TJ, Crosa JH (2007) Transcription termination within the iron transport-biosynthesis operon of *Vibrio anguillarum* requires an antisense RNA. *J Bacteriol* **189**: 3479-3488
- Storz G, Vogel J, Wassarman KM (2011) Regulation by small RNAs in bacteria: expanding frontiers. *Mol Cell* **43**: 880-891
- Suzuki K, Babitzke P, Kushner SR, Romeo T (2006) Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E. *Genes Dev* **20**: 2605-2617
- Svenningsen SL, Tu KC, Bassler BL (2009) Gene dosage compensation calibrates four regulatory RNAs to control *Vibrio cholerae* quorum sensing. *EMBO J* **28**: 429-439
- Svenningsen SL, Waters CM, Bassler BL (2008) A negative feedback loop involving small RNAs accelerates *Vibrio cholerae*'s transition out of quorum-sensing mode. *Genes Dev* **22**: 226-238
- Tanabe T, Funahashi T, Nakao H, Maki J, Yamamoto S (2013) The *Vibrio parahaemolyticus* small RNA RyhB promotes production of the siderophore vibrioferrin by stabilizing the polycistronic mRNA. *J Bacteriol* **195**: 3692-3703
- Tanabe T, Miyamoto K, Tsujibo H, Yamamoto S, Funahashi T (2015) The small RNA Spot 42 regulates the expression of the type III secretion system 1 (T3SS1) chaperone protein VP1682 in *Vibrio parahaemolyticus*. *FEMS Microbiol Lett* **362**
- Taylor RK, Miller VL, Furlong DB, Mekalanos JJ (1987) Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proceedings of the National Academy of Sciences of the United States of America* **84**: 2833-2837
- Thomas S, Williams SG, Manning PA (1995) Regulation of tcp genes in classical and El Tor strains of *Vibrio cholerae* O1. *Gene* **166**: 43-48

Thomason MK, Bischler T, Eisenbart SK, Forstner KU, Zhang A, Herbig A, Nieselt K, Sharma CM, Storz G (2015) Global transcriptional start site mapping using differential RNA sequencing reveals novel antisense RNAs in *Escherichia coli*. *J Bacteriol* **197**: 18-28

Thompson FL, Iida T, Swings J (2004) Biodiversity of vibrios. *Microbiol Mol Biol Rev* **68**: 403-431, table of contents

Toffano-Nioche C, Luo Y, Kuchly C, Wallon C, Steinbach D, Zytnicki M, Jacq A, Gautheret D (2013) Detection of non-coding RNA in bacteria and archaea using the DETR'PROK Galaxy pipeline. *Methods* **63**: 60-65

Toffano-Nioche C, Nguyen AN, Kuchly C, Ott A, Gautheret D, Bouloc P, Jacq A (2012) Transcriptomic profiling of the oyster pathogen *Vibrio splendidus* opens a window on the evolutionary dynamics of the small RNA repertoire in the *Vibrio* genus. *RNA* **18**: 2201-2219

Tojo S, Satomura T, Morisaki K, Deutscher J, Hirooka K, Fujita Y (2005) Elaborate transcription regulation of the *Bacillus subtilis* *ilv-leu* operon involved in the biosynthesis of branched-chain amino acids through global regulators of CcpA, CodY and TnrA. *Molecular Microbiology* **56**: 1560-1573

Tojo S, Satomura T, Morisaki K, Yoshida K-I, Hirooka K, Fujita Y (2004) Negative Transcriptional Regulation of the *ilv-leu* Operon for Biosynthesis of Branched-Chain Amino Acids through the *Bacillus subtilis* Global Regulator TnrA. *Journal of Bacteriology* **186**: 7971-7979

Tomizawa J, Itoh T, Selzer G, Som T (1981) Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proceedings of the National Academy of Sciences of the United States of America* **78**: 1421-1425

Trotochaud AE, Wassarman KM (2004) 6S RNA function enhances long-term cell survival. *J Bacteriol* **186**: 4978-4985

Trotochaud AE, Wassarman KM (2005) A highly conserved 6S RNA structure is required for regulation of transcription. *Nature structural & molecular biology* **12**: 313-319

Trotonda MP, Tamber S, Memmi G, Cheung AL (2008) MgrA represses biofilm formation in *Staphylococcus aureus*. *Infect Immun* **76**: 5645-5654

Updegrove TB, Shabalina SA, Storz G (2015) How do base-pairing small RNAs evolve? *FEMS Microbiol Rev* **39**: 379-391

Updegrove TB, Zhang A, Storz G (2016) Hfq: the flexible RNA matchmaker. *Curr Opin Microbiol* **30**: 133-138

- Urban JH, Vogel J (2007) Translational control and target recognition by Escherichia coli small RNAs in vivo. *Nucleic Acids Res* **35**: 1018-1037
- Urban JH, Vogel J (2008) Two seemingly homologous noncoding RNAs act hierarchically to activate glmS mRNA translation. *PLoS Biol* **6**: e64
- Urban JH, Vogel J (2009) A green fluorescent protein (GFP)-based plasmid system to study post-transcriptional control of gene expression in vivo. *Methods Mol Biol* **540**: 301-319
- Vakulskas CA, Potts AH, Babitzke P, Ahmer BM, Romeo T (2015) Regulation of bacterial virulence by Csr (Rsm) systems. *Microbiol Mol Biol Rev* **79**: 193-224
- Val ME, Skovgaard O, Ducos-Galand M, Bland MJ, Mazel D (2012) Genome engineering in Vibrio cholerae: a feasible approach to address biological issues. *PLoS Genet* **8**: e1002472
- van der Meulen SB, de Jong A, Kok J (2016) Transcriptome landscape of Lactococcus lactis reveals many novel RNAs including a small regulatory RNA involved in carbon uptake and metabolism. *RNA Biol* **13**: 353-366
- Vanderpool CK, Gottesman S (2004) Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. *Mol Microbiol* **54**: 1076-1089
- Vanhove AS, Duperthuy M, Charriere GM, Le Roux F, Goudenege D, Gourbal B, Kieffer-Jaquinod S, Coute Y, Wai SN, Destoumieux-Garzon D (2015) Outer membrane vesicles are vehicles for the delivery of Vibrio tasmaniensis virulence factors to oyster immune cells. *Environmental microbiology* **17**: 1152-1165
- Vanhove AS, Rubio TP, Nguyen AN, Lemire A, Roche D, Nicod J, Vergnes A, Poirier AC, Disconzi E, Bachere E, Le Roux F, Jacq A, Charriere GM, Destoumieux-Garzon D (2016) Copper homeostasis at the host vibrio interface: lessons from intracellular vibrio transcriptomics. *Environmental microbiology* **18**: 875-888
- Verdon J, Girardin N, Lacombe C, Berjeaud JM, Hechard Y (2009) delta-hemolysin, an update on a membrane-interacting peptide. *Peptides* **30**: 817-823
- Vesth T, Wassenaar TM, Hallin PF, Snipen L, Lagesen K, Ussery DW (2010) On the origins of a Vibrio species. *Microb Ecol* **59**: 1-13
- Vezzulli L, Colwell RR, Pruzzo C (2013) Ocean warming and spread of pathogenic vibrios in the aquatic environment. *Microb Ecol* **65**: 817-825

Vidal-Ingigliardi D, Richet E, Raibaud O (1991) Two MalT binding sites in direct repeat. A structural motif involved in the activation of all the promoters of the maltose regulons in *Escherichia coli* and *Klebsiella pneumoniae*. *J Mol Biol* **218**: 323-334

Vitreschak AG, Lyubetskaya EV, Shirshin MA, Gelfand MS, Lyubetsky VA (2004) Attenuation regulation of amino acid biosynthetic operons in proteobacteria: comparative genomics analysis. *FEMS Microbiol Lett* **234**: 357-370

Vogel J (2003) RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. *Nucleic Acids Research* **31**: 6435-6443

Wadler CS, Vanderpool CK (2007) A dual function for a bacterial small RNA: SgrS performs base pairing-dependent regulation and encodes a functional polypeptide. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 20454-20459

Wagner EG, Romby P (2015a) Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. *Adv Genet* **90**: 133-208

Wagner EGH, Romby P (2015b) Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. In *Advances in genetics* Vol. 90, pp 133-208. Elsevier

Waldor MK, Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**: 1910-1914

Wang L, Wang W, Li F, Zhang J, Wu J, Gong Q, Shi Y (2015) Structural insights into the recognition of the internal A-rich linker from OxyS sRNA by *Escherichia coli* Hfq. *Nucleic Acids Res* **43**: 2400-2411

Wang X, Dubey AK, Suzuki K, Baker CS, Babitzke P, Romeo T (2005) CsrA post-transcriptionally represses *pgaABCD*, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. *Mol Microbiol* **56**: 1648-1663

Wassarman KM (2018) 6S RNA, a Global Regulator of Transcription. *Microbiol Spectr* **6**

Wassarman KM, Repoila F, Rosenow C, Storz G, Gottesman S (2001) Identification of novel small RNAs using comparative genomics and microarrays. *Genes & development* **15**: 1637-1651

Wassarman KM, Saecker RM (2006) Synthesis-mediated release of a small RNA inhibitor of RNA polymerase. *Science* **314**: 1601-1603

Wassarman KM, Storz G (2000) 6S RNA regulates *E. coli* RNA polymerase activity. *Cell* **101**: 613-623

Wassarman KM, Zhang A, Storz G (1999) Small RNAs in *Escherichia coli*. *Trends Microbiol* **7**: 37-45

Waters CM, Lu W, Rabinowitz JD, Bassler BL (2008) Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of *vpsT*. *J Bacteriol* **190**: 2527-2536

Weber B, Lindell K, El Qaidi S, Hjerde E, Willassen NP, Milton DL (2011) The phosphotransferase VanU represses expression of four *qrr* genes antagonizing VanO-mediated quorum-sensing regulation in *Vibrio anguillarum*. *Microbiology* **157**: 3324-3339

Weber GG, Kortmann J, Narberhaus F, Klose KE (2014) RNA thermometer controls temperature-dependent virulence factor expression in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America* **111**: 14241-14246

Wek RC, Hatfield GW (1986) Nucleotide sequence and in vivo expression of the *ilvY* and *ilvC* genes in *Escherichia coli* K12. Transcription from divergent overlapping promoters. *J Biol Chem* **261**: 2441-2450

Wek RC, Hauser CA, Hatfield GW (1985) The nucleotide sequence of the *ilvBN* operon of *Escherichia coli*: sequence homologies of the acetohydroxy acid synthase isozymes. *Nucleic acids research* **13**: 3995-4010

Wen J, Won D, Fozo EM (2014) The ZorO-OrzO type I toxin-antitoxin locus: repression by the OrzO antitoxin. *Nucleic Acids Res* **42**: 1930-1946

Wessler SR, Calvo JM (1981) Control of *leu* operon expression in *Escherichia coli* by a transcription attenuation mechanism. *J Mol Biol* **149**: 579-597

Whiteley M, Diggle SP, Greenberg EP (2017) Progress in and promise of bacterial quorum sensing research. *Nature* **551**: 313-320

Willins DA, Calvo JM (1992) In vitro transcription from the *Escherichia coli* *ilvIH* promoter. *J Bacteriol* **174**: 7648-7655

Wilusz CJ, Wilusz J (2013) Lsm proteins and Hfq: Life at the 3' end. *RNA Biol* **10**: 592-601

Winkler WC, Cohen-Chalamish S, Breaker RR (2002) An mRNA structure that controls gene expression by binding FMN. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 15908-15913

Wright PR, Georg J, Mann M, Sorescu DA, Richter AS, Lott S, Kleinkauf R, Hess WR, Backofen R (2014) CopraRNA and IntaRNA: predicting small RNA targets, networks and interaction domains.

Xie ZY, Hu CQ, Chen C, Zhang LP, Ren CH (2005) Investigation of seven *Vibrio* virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong, China. *Lett Appl Microbiol* **41**: 202-207

Yakhnin AV, Baker CS, Vakulskas CA, Yakhnin H, Berezin I, Romeo T, Babitzke P (2013) CsrA activates flhDC expression by protecting flhDC mRNA from RNase E-mediated cleavage. *Mol Microbiol* **87**: 851-866

Yamaichi Y, Iida T, Park KS, Yamamoto K, Honda T (1999) Physical and genetic map of the genome of *Vibrio parahaemolyticus*: presence of two chromosomes in *Vibrio* species. *Mol Microbiol* **31**: 1513-1521

Yamamoto S, Izumiya H, Mitobe J, Morita M, Arakawa E, Ohnishi M, Watanabe H (2011) Identification of a chitin-induced small RNA that regulates translation of the tfoX gene, encoding a positive regulator of natural competence in *Vibrio cholerae*. *J Bacteriol* **193**: 1953-1965

Yanofsky C (1981) Attenuation in the control of expression of bacterial operons. *Nature* **289**: 751-758

Yanofsky C (2000) Transcription attenuation: once viewed as a novel regulatory strategy. *J Bacteriol* **182**: 1-8

Yanofsky C (2001) Advancing our knowledge in biochemistry, genetics, and microbiology through studies on tryptophan metabolism. *Annual review of biochemistry* **70**: 1-37

Zere TR, Vakulskas CA, Leng Y, Pannuri A, Potts AH, Dias R, Tang D, Kolaczowski B, Georgellis D, Ahmer BM, Romeo T (2015) Genomic Targets and Features of BarA-UvrY (-SirA) Signal Transduction Systems. *PLoS One* **10**: e0145035

Zhang A, Schu DJ, Tjaden BC, Storz G, Gottesman S (2013) Mutations in interaction surfaces differentially impact *E. coli* Hfq association with small RNAs and their mRNA targets. *J Mol Biol* **425**: 3678-3697

Zhang A, Wassarman KM, Rosenow C, Tjaden BC, Storz G, Gottesman S (2003) Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol* **50**: 1111-1124

Zhang MG, Liu JM (2019) Transcription of cis-antisense small RNA MtlS in *Vibrio cholerae* is regulated by transcription of its target gene mtlA. *J Bacteriol*: JB.00178-00119

Zhang Y, Yan D, Xia L, Zhao X, Osei-Adjei G, Xu S, Sheng X, Huang X (2017a) The malS-5'UTR regulates hisG, a key gene in the histidine biosynthetic pathway in *Salmonella enterica* serovar Typhi.

*Can J Microbiol* **63**: 287-295

Zhang YJ, Chen G, Lin H, Wang P, Kuang B, Liu J, Chen S (2017b) Development of a regulatable expression system for the functional study of *Vibrio vulnificus* essential genes. *Antonie Van Leeuwenhoek* **110**: 607-614

Zhao X, Koestler BJ, Waters CM, Hammer BK (2013) Post-transcriptional activation of a diguanylate cyclase by quorum sensing small RNAs promotes biofilm formation in *Vibrio cholerae*. *Mol Microbiol* **89**: 989-1002

Zhao Z, Chen C, Hu CQ, Ren CH, Zhao JJ, Zhang LP, Jiang X, Luo P, Wang QB (2010) The type III secretion system of *Vibrio alginolyticus* induces rapid apoptosis, cell rounding and osmotic lysis of fish cells. *Microbiology* **156**: 2864-2872

Zhao Z, Liu J, Deng Y, Huang W, Ren C, Call DR, Hu C (2018) The *Vibrio alginolyticus* T3SS effectors, Val1686 and Val1680, induce cell rounding, apoptosis and lysis of fish epithelial cells. *Virulence* **9**: 318-330

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**: 3406-3415



**Titre :** Rôles des ARN régulateurs chez des vibrios pathogènes d'espèces d'intérêt aquacole

**Mots clés :** vibrio, métabolisme bactérien, ARNs, maltose,

**Résumé :** Les petits ARN régulateurs bactériens, impliqués dans la voie de la glycolyse/néoglucogenèse, Fbp et PpsA et cet ARNm de *fbp* étaient une cible directe de Vsr217. J'ai également exploré la régulation de la biosynthèse des acides aminés à chaîne ramifiée (BCAA: Leucine, Valine et Isoleucine) chez *V. alginolyticus*, un agent pathogène des poissons et mollusques et des poissons de mer et un agent pathogène humain émergent opportuniste. Nous avons constaté que l'opéron *ilvGMEDA* (codant la voie principale pour la biosynthèse des BCAAs) est régulé par un peptide leader traduit. Ainsi, la traduction d'un peptide riche en BCAA codé en amont des gènes de structure fournit une réponse adaptative par un mécanisme similaire au modèle canonique de *E. coli*. Cette étude portant sur un organisme non modèle à Gram-négatif met en évidence la conservation mécanistique de l'atténuation de la transcription malgré l'absence de conservation de la séquence primaire.

*V. tasmaniensis* LGP32 est un pathogène de l'huître facultatif. Un ARNs Vsr217 s'est révélé être conservé dans les vibrios et fortement régulé à la hausse lors de l'infection des huîtres. J'ai trouvé que *vsr217* et le gène en aval *malK* (codant pour une sous-unité du transporteur principal de maltose) sont tous deux exprimés à partir d'un promoteur en amont régulé par l'activateur de maltose MalT, Vsr217 étant généré à partir de la longue 5' UTR de l'ARNm de *malK*. Outre un effet cis sur l'expression du *malK*, qui diminue chez le mutant  $\Delta vsr217$ , nous avons constaté que l'absence de cet ARNs entraînait, lors de la croissance de cellules dans du maltose, l'augmentation de deux enzymes importantes

**Title :** Roles of regulatory RNAs in vibrios pathogenic to species of aquaculture interest

**Keywords :** vibrio, bacterial metabolism, sRNA, maltose,

**Abstract:** Bacterial regulatory small RNAs, usually 50-300 nt long, act by base-pairing with specific mRNA targets, affecting their translation and/or stability, are important elements which regulate a variety of processes. *V. tasmaniensis* LGP32 is a facultative oyster pathogen. A sRNA Vsr217 was found to be conserved within vibrios and highly upregulated during oyster infection. I found that *vsr217* and the downstream gene *malK* (encoding a subunit of the major maltose transporter) are both expressed from an upstream promoter regulated by the maltose activator MalT with Vsr217 being generated from the long 5' UTR of the *malK* mRNA. Beside a cis-effect on *malK* expression, which decreases in the  $\Delta vsr217$  mutant, we found that the absence of this sRNA resulted, when cells grown in maltose, in the increase of two important enzymes involved

in the glycolysis/neoglucogenesis pathway, Fbp and PpsA and that *fbp* mRNA was a direct target of Vsr217. I also explored the regulation of the biosynthesis of branched-chain amino acids (BCAAs: Leucine, Valine and Isoleucine) in *V. alginolyticus*, a marine fish and shellfish pathogen and an emerging opportunistic human pathogen. We found that the *ilvGMEDA* operon (encoding the main pathway for biosynthesis of BCAAs) is regulated by a translated leader peptide. Thus, the translation of a BCAA rich peptide encoded upstream of the structural genes provides an adaptive response by a mechanism similar to the *E. coli* canonical model. This study with a non-model Gram-negative organism highlights the mechanistic conservation of transcription attenuation despite the absence of primary sequence conservation.