A Small Regulatory RNA Generated from the malK 5′ Untranslated Region Targets Gluconeogenesis in Vibrio Species

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ABSTRACT

Vsr217 is a small RNA from Vibrio tasmaniensis LGP32, a pathogen associated with mortality events affecting juvenile oysters. The vsr217 gene is located within the 5′ untranslated region (UTR) of malK, encoding the ATPase component of the maltose importer, and is conserved within the genus Vibrio. In the presence of maltose, vsr217 is regulated by MalT, the positive regulator of the maltose regulon. vsr217 is required in cis for the full expression of malK. In addition, Vsr217 acts in trans to downregulate the expression of fbp encoding fructose-1,6-bisphosphatase, an enzyme involved in gluconeogenesis. Thus, in the presence of maltose, the induction of Vsr217 is expected to promote glycolysis by negatively regulating the expression of a key enzyme of gluconeogenesis.

IMPORTANCE

Juvenile pacific oysters have been subject in recent years to summer mortality episodes with deep economic consequences. The pathogen Vibrio tasmaniensis has been associated with such mortality events. For bacterial pathogens, survival within the host requires profound metabolic adaptations according to available resources. All kinds of regulatory elements, including noncoding RNAs, orchestrate this response. Oysters are rich in glycogen, a precursor of maltose, and we previously reported that V. tasmaniensis maltose-regulated genes are strongly induced during oyster infection. Here, we report the dual mechanism by which a small regulatory RNA, generated from the 5′ untranslated region of a gene belonging to the maltose regulon, acts both in cis and trans. In cis, it stimulates growth on maltose, and in trans, it downregulates the expression of a gene associated with gluconeogenesis, thus coordinating maltose utilization with central carbon metabolism.

KEYWORDS

Vibrio, sRNA, 5′ UTR, maltose regulon, gluconeogenesis

In the last 20 years, the importance of noncoding RNAs (sRNAs) in bacterial regulatory networks has become widely recognized. The development of genome-wide transcriptomic studies has led to the exploration of transcriptomic landscapes of nonmodel bacterial organisms, either pathogens or those of environmental interest, leading to the discovery of many sRNAs (for reviews, see references 1–3). While classical bona fide sRNAs are expressed from intergenic regions, a newly recognized class of sRNAs includes sRNAs generated from 5′ and 3′ untranslated regions (UTRs) of coding genes that act in trans on genetically unlinked targets. A genome-wide search of small regulatory RNAs in Escherichia coli led to the identification of several sRNAs expressed from 5′ or 3′ UTRs (4), but only a few of them, mostly stemming from 3′ UTRs, have been characterized. For instance, CpxQ is generated from the 3′ UTR of cpxP mRNA, which encodes a periplasmic chaperone involved in the inner membrane stress response. CpxQ was shown to repress several inner membrane proteins, thus mitigating membrane stress (5). Another example is MicL, which is transcribed from a σ54-dependent promoter internal to the upstream protein coding sequence, and then cleaved to a shorter product to act in cis to regulate the expression of the major outer membrane lipoprotein Lpp (6). A few examples are associated with Vibrio species. In Vibrio
*Vibrio tasmaniensis* LGP32 (previously *V. splendidus* LGP32) is a member of the *V. splendidus* clade (14) that has been associated with summer mortalities of juvenile Pacific oysters *Crassostrea gigas* (15). It is a facultative intracellular pathogen of oysters, invading the oyster hemocytes, the host immune cells (16–18). A genome-wide search for noncoding sRNAs in *V. tasmaniensis* LGP32 by transcriptome sequencing (RNA-seq) led us to identify 250 potential sRNAs, among which, several were confirmed by Northern blots and reverse transcription-PCRs (RT-PCRs) (19). More recently, a transcriptomic analysis of LGP32 within the host revealed that vsr217 was one of the most highly induced genes compared to that during growth under seawater conditions (18). Interestingly, vsr217 is located upstream of *malK*, a gene encoding the ATPase subunit of the maltose transporter, a member of the ABC transporter family (reviewed in reference 20). In addition, the maltose regulon was highly induced in oysters, a metabolic switch that can be related to the fact that oysters are rich in glycogen, a precursor of maltose (18).

Here, we show that vsr217 is a member of the maltose regulon and is positively controlled by the transcriptional regulator MalT. While, within the 5′ UTR of *malK* mRNA, the Vsr217 sequence acts in cis to stabilize *malK* mRNA, it also generates an sRNA that acts in trans to repress the expression of a key gene involved in gluconeogenesis.

**RESULTS**

The genetic organization of vsr217-*malK* is conserved in different *Vibrio* species. Vsr217 is an sRNA from *V. tasmaniensis* expressed from the *malK* 5′ UTR. We investigated its conservation within the *Vibrio* genus by sequence comparison of *malK* upstream sequences from species representative of different *Vibrio* clades (14) (Fig. 1). Five regions are especially conserved. Region I is immediately upstream of a predicted −35 promoter sequence (P1). Region II overlaps with the −10 sequence of the P1 promoter and includes a putative MalT operator. MalT is the positive regulator of the maltose regulon in *E. coli* and other *Enterobacteriaceae* (21) which binds to MalT consensus sequences (22). Region III corresponds to the 5′ end of Vsr217 (see below) and includes the 32 first nucleotides of the sRNA. Region IV contains a conserved TTTTC motif present at the 3′ end of Vsr217. Region V is the largest conserved sequence and contains a second predicted α70 promoter (P2) as well as the ribosome binding site for *malK*. No Rho-independent terminator was predicted between the 3′ end of vsr217 and *malK*. This analysis highlights the conservation of a large putative regulatory region upstream of *malK* in *Vibrio*. 
Vsr217 is induced by maltose in a MalT-dependent manner and is generated from the 5' UTR of \textit{malK}.

The conserved localization of \textit{vsr217} upstream of \textit{malK} and the presence of a MalT box overlapping the putative P1 promoter of \textit{vsr217} (Fig. 1) suggested that Vsr217 is a member of the maltose regulon controlled by MalT. The production of Vsr217 and \textit{malK} mRNA was tested by Northern blots in the presence or absence of maltose (Fig. 2A). Both Vsr217 and \textit{malK} mRNA were strongly induced by maltose in a MalT-dependent manner.

\textbf{FIG 1} Vsr217 is conserved among \textit{Vibrio} strains. The upstream regions of the \textit{malK} gene of several \textit{Vibrio} strains belonging to different clades (14) were aligned using Muscle (39). For reasons of space, a less-conserved sequence between regions III and IV is not shown, as indicated by two slashes. Conserved nucleotides are indicated by * under the alignment. \textit{V. tasmaniensis} sequence is in red, with the \textit{vsr217} sequence in italic font. Important features are in boldface font, including \textit{Vsr217} 5' and 3' ends; 235 and 210 regions of promoters predicted using BPROM (see Materials and Methods) are underlined. \textit{malK} initiation codon is indicated in boldface red font.

Vsr217 is induced by maltose in a MalT-dependent manner and is generated from the 5' UTR of \textit{malK}. The conserved localization of \textit{vsr217} upstream of \textit{malK} and the presence of a MalT box overlapping the putative P1 promoter of \textit{vsr217} (Fig. 1) suggested that Vsr217 is a member of the maltose regulon controlled by MalT. The production of Vsr217 and \textit{malK} mRNA was tested by Northern blots in the presence or absence of maltose (Fig. 2A). Both Vsr217 and \textit{malK} mRNA were strongly induced by maltose in a MalT-dependent manner.
maltose, whereas no expression was detected in the presence of glucose. Vsr217 and malK mRNA were not detected in the malT mutant, indicating that their expression is MalT dependent. Under the maltose condition, a high-molecular-weight band was detected with both vsr217 and malK probes, indicating the existence of a large transcript encompassing the two genes. A smaller band was detected with the malK probe but not with the vsr217 probe, indicating the existence of a transcript including malK RNA without Vsr217. The transcription of malK was also tested in a mutant harboring a complete deletion of the vsr217 sequence (see below and Fig. 2B). Using the malK probe, a single band was observed whose size was compatible with that of a malK transcript originating from the putative promoter P1, shortened by the absence of vsr217 (Fig. 1A). Of note, malK mRNA quantity was reduced in the Δvsr217 mutant compared to that in its parental strain (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 supplemented with glucose or maltose as indicated. (A, top left) Northern blots of malK in LGP32 WT, Δvsr217, and ΔmalT strains, using a malK probe. tmRNA was used for normalization. (Bottom left) Same membrane using a probe complementary to Vsr217. (A, right) Quantification of malK mRNA by ImageJ software. Error bars correspond to the standard errors of the means (n = 3). Significance was determined using a Student’s unpaired t test. **, P < 0.01. (B) Determination of 5’ and 3’ ends of Vsr217 and malK mRNA of cells grown in maltose. In each case, 20 clones were analyzed. The genetic organization is represented on top (not drawn to scale). Approximate position of primers used for RT-PCR is indicated (F and R). (C) Evidence for the existence of a Vsr217-malK transcript by RT-PCR using LGP32 WT total RNA extracted from cultures in either glucose or maltose as indicated.
The 5’ and 3’ ends of transcripts from the vsr217-malK locus were determined by 5’ and 3’ rapid amplification of cDNA ends (RACE) mappings (Fig. 2B). For Vsr217, all clones displayed the same extremities. The Vsr217 5’ end was compatible with a transcription stemming from the P1 promoter, while the transcript terminated at the level of a conserved TTTTC motif. In contrast, for malK mRNA, if the 3’ end was unique, due to the presence of a Rho-independent terminator, different malK 5’ ends were identified. The majority of them (57.5%) corresponded to a transcription start site from the P2 promoter. Other 5’ end sequences mapped upstream of P2, some of them within vsr217. Among them, 6% had a 5’ end corresponding to a transcription initiation from P1, hence including the whole Vsr217 sequence (Fig. 2B). The existence of a vsr217-malK transcript was also confirmed by RT-PCR, whose amount increased in the presence of maltose (Fig. 2C).

We concluded that Vsr217 originates from the malK P1 promoter. The existence of transcripts encompassing Vsr217-malK mRNA suggested that Vsr217 is generated from malK 5’ UTR and that processing of the 5’ end of this larger transcript generates malK transcripts.

Deletion of vsr217 results in malK mRNA instability. The amount of malK mRNA was reduced in the absence of vsr217 (Fig. 2A), possibly because of malK mRNA destabilization. To check this, the half-life of Vsr217 and malK and vsr217-malK transcripts was determined in the wild-type (WT) and Δvsr217 strains (Fig. 3A and B). Vsr217 was a stable sRNA (half-life \( t_{1/2} \), ~7.5 min), whereas malK and vsr217-malK transcripts were unstable \( t_{1/2} \), ~30 s). Deletion of vsr217 resulted in an even more unstable malK transcript \( t_{1/2} \), ~15 s).

The 5’ end of the malK mRNA in the Δvsr217 mutant was determined by 5’ RACE. In contrast to the parental strain, the majority of the malK transcripts (64.2%) in the Δvsr217 mutant had a 5’ end generated from the P1 promoter, whereas only 21.4% corresponded to transcripts generated from the P2 promoter (Fig. 3C).

Does the malK mRNA level reduction in the Δvsr217 mutant affect growth in the presence of maltose as the sole carbon source? Indeed, when grown in minimal medium supplemented with maltose, the Δvsr217 mutant had a lower growth rate than its parental strain. This difference was not observed in minimal medium supplemented with glucose (see Fig. S1 in the supplemental material). The maltose-dependent growth defect of the Δvsr217 mutant was corrected by the presence of a plasmid expressing malK only but not by a plasmid expressing vsr217 under the P1 promoter (Table 1). We concluded that the growth defect in the mutant is due to the cis effect of the deletion of vsr217 on malK mRNA stability, leading to the decrease of the message.

A U-rich terminal motif of Vsr217 is a stabilizing factor for Vsr217 but not for malK mRNA. Vsr217 contains a conserved U-rich motif at its 3’ end (Fig. 1A). We hypothesized that it could play a role in generating Vsr217, either by promoting transcription termination, by being a processing site, or by contributing to its stability. To test this, we constructed a vsr217 mutant lacking its 13 last nucleotides (ΔpolyT) and checked by Northern blotting how the deletion would affect the amounts of Vsr217, malK mRNA, and their precursor (Fig. 4A).

The absence of the last 13 nucleotides (ΔpolyT mutant) led to a 100-fold reduction of Vsr217 compared to that in the WT strain, whereas the amount of malK mRNA was not affected in contrast to what was observed in the Δvsr217 mutant. Consistent with this result, we observed that malK mRNA stability did not decrease in the ΔpolyT mutant compared to that in the WT strain, contrary to Vsr217 stability (Fig. 4B). Surprisingly, the quantity of the precursor transcript vsr217-malK mRNA was also not affected in the ΔpolyT mutant as could have been expected if the deleted terminal motif was involved in a processing event generating Vsr217 and malK mRNA. Altogether, our results suggest that the U-rich terminal motif is involved in Vsr217 stability but not in malK mRNA stability.

In addition, no growth difference was observed between the ΔpolyT mutant and its parental strain in minimal medium supplemented with either glucose or maltose.
Fig. 3 Deleting vsr217 leads to an instability of malK mRNA. (A) Amounts of malK mRNA and Vsr217 from cells growing in minimal medium supplemented with maltose (OD600 ~ 0.4) were determined by Northern blotting over time after adding rifampicin to block transcription. (B) Kinetics of degradation of malK mRNA and Vsr217 determined from the experiment presented in panel A. (C) 5’ ends of the malK mRNAs in the Δvsr217 mutant.

(Fig. 4C), confirming that the growth defect observed in the Δvsr217 mutant was due to a cis effect on malK expression and not to the absence of Vsr217 production.

Vsr217 affects the expression of fbp, a gluconeogenic enzyme. The existence of a stable, abundant Vsr217 sRNA suggests a trans-acting function on targets possibly not directly related to maltose utilization but nevertheless regulated in response to maltose. Putative Vsr217 targets were searched by identifying proteins whose expression levels varied between the WT and Δvsr217 strains, especially when grown in maltose. Using a proteomic mass spectrometry approach, 2,104 proteins from 4,371 coding DNA sequences (CDSs) harbored by the V. tasmaniensis genome were identified. In glucose, the protein contents were the same between the two strains. However, in maltose, the amounts of 22 proteins were significantly altered (fold change of >2 or <0.5, P value < 0.05) in the mutant, with 11 increasing and 11 decreasing (Table 2). Among them, MalK decreased in the Δvsr217 mutant, consistent with the reduced amount of malK mRNA (Fig. 2).

Among these 22 potential targets of Vsr217, to identify candidates for direct mRNA targets, we used the software CopraRNA, which predicts sRNA targets and their pairing regions with the sRNA by comparative genomics (23). From the list of proteins whose
levels were affected in the Δvsr217 mutant, only one came out in the list of the first 100 best candidates for direct interaction: VS_2771, ranked 8th by CopraRNA, corresponds to Fbp, a class 1 fructose-1,6-bisphosphatase that catalyzes the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate, a rate-limiting step of the gluconeogenesis pathway (Table 2). Fbp expression increased in the Δvsr217 mutant, suggesting that Vsr217 could be a negative regulator of fbp.

Since posttranscriptional inhibition by sRNAs often leads to a stability reduction of target mRNAs (24), we determined by Northern blotting the amount of fbp mRNA in the Δvsr217 mutant, which has a drastically reduced amount of Vsr217 but still retains a wild-type expression level of malK, a difference that was abolished in the mutant, suggesting that Vsr217 could be a negative regulator of fbp.

**fbp mRNA is a direct target of Vsr217.** A bioinformatics analysis using IntaRNA identified a potential base-pairing region between Vsr217 and fbp mRNA, ending 29 nucleotides (nt) upstream of the fbp initiation codon (Fig. 6A). To confirm a putative direct interaction, the Vsr217 trans activity on fbp expression was tested in *E. coli* using a two-plasmid-based reporter system (25, 26). The first plasmid carried the vsr217 gene cloned under a P_LlacO promoter (pPlac-Vsr217). The second plasmid contained an in-frame gene fusion between the predicted pairing region of fbp mRNA including initiation codon and the second codon of the gfp CDS (Table 1). The reporter fusion was cloned under the control of a P_LlacO promoter, which is constitutive in the absence of a TetR repressor. pPtet-Fbp-GFP was introduced in *E. coli* together with pPlac-Vsr217, and the effect of Vsr217 production on the expression of fbp was monitored via fluorescence measurements (see Materials and Methods).

The presence of pPlac-Vsr217 plasmid led to a 20% reduction in the expression of the fbp-GFP fusion compared to that in the strain containing a control empty plasmid

**TABLE 1 Strains and plasmids used in this study**

| Strain or plasmid | Relevant characteristics | Reference or source |
|-------------------|-------------------------|---------------------|
| Strains           |                         |                     |
| V. tasmaniensis   |                         |                     |
| LGP32             | Pathogen of oyster *C. gigas* | 40 |
| Δvsr217          | LGP32 carrying a deletion of vsr217 | This study |
| ΔmalT            | LGP32 carrying a deletion of malT | This study |
| ΔpolyT          | LGP32 carrying a deletion of polyT at the 3' end of vsr217 | This study |
| E. coli          |                         |                     |
| DHS5a            | F− endA1 glnIV4 thi-1 recA1 relA1 gyrA96 deoR napG purB20 ϕ80d lacZΔM15 Δ(lacZYA-argF)U169 hsrd17 (r− m−) λ− | Lab collection |
| MG1655           | F− λ− ivG− rfb-50 rph-1 | Lab collection |
| MG1655 Δhfq      | F− λ− ivG− rfb-50 rph-1 Δhfq | 41 |
| II3813           | *E. coli* K-12 lacIQ thi1 supE44 endA1 recA1 hsrd17 gyrA462 zei298::tn10 [Tc] ΔthyA::[erm-pir116][Er]; cloning host | 42 |
| GEB883           | *E. coli* K-12 Δdapa::erm pir [Er] RP4-2 ΔrecA gyrA462 zei298::Tn10 [Tc]; donor strain for conjugation | 34 |
| Plasmids         |                         |                     |
| pSW7848          | Suicide vector with an R6K origin, requiring the Pir protein for its replication, pBAD-cccB, Cm' | 35, 43 |
| pSW7848-Δvsr217  | pSW7848 carrying the mutant allele Δvsr217, Cm' | This study |
| pSW7848-ΔmalT    | pSW7848 carrying an in-frame deletion of malK, Cm' | This study |
| pSW7848-ΔpolyT   | pSW7848 carrying the polyT 13-nt deletion at the 3' end of vsr217, Cm' | This study |
| pGEB12           | Replicative, pSU18 with oriT15A, oriT<sup>tp</sup>, Cm' | 44 |
| pP1-P2-MalK      | pGEB12, Cm', malK under the control of P1-P2 promoters | This study |
| pP1-Vsr217       | pGEB12, Cm', vsr217 under the control of vsr217-malK P1 promoter | This study |
| pZE12MCS         | Based on pZE12-Luc, P_Ltet<sub>0</sub> ColE1 Ori1, Amp<sup>+</sup> | 26 |
| pPlac-Vsr217     | pZE12MCS, P_Ltet<sub>0</sub>-vsr217, Amp<sup>+</sup> | This study |
| pXG-10           | pSC101<sup>*</sup> origin of replication (low copy), P_Ltet<sub>0</sub> promoter, gfp<sup>+</sup>, Cm' | 26 |
| pPtet-Fbp-GFP    | pXG-10 [−125, +3]-fbp::gfp, Cm' | This study |
| pXG-10∆alp<sub>φ</sub> | pXG-10, ∆alp<sub>φ</sub>, Cm' | This study |

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Expression was further reduced to ~40% when isopropyl-β-D-thiogalactopyranoside (IPTG) was added to fully induce vsr217 expression.

The RNA chaperone Hfq is often required for the interaction of sRNAs with their targets, including in *Vibrio* (27–29). To determine whether the potential interaction (Fig. 6B).
### Table 2: Proteins whose level is affected by Vsr217 in maltose

| Locus tag | Fasta header | No. of peptides | Fold change Δvsr217/WT in Mal | P value | Fold change Δvsr217/WT in Glu | P value | Rank | CopraRNA | FDRa |
|-----------|--------------|-----------------|-------------------------------|---------|-------------------------------|---------|------|----------|------|
| **Upregulated proteins** | | | | | | | | | |
| VS_II0899 | Prolyl aminopeptidase | 10 | 6.81 | 0.00 | 0.88 | 0.89 | >100 | >0.23 |
| VS_II0126 | Oxidoreductase | 8 | 5.84 | 0.05 | 1.00 | NaN | >100 | >0.23 |
| VS_II0346 | Glyceraldehyde-3-phosphate dehydrogenase | 18 | 3.33 | 0.01 | 1.11 | 0.81 | >100 | >0.23 |
| | GapC | | | | | | | | |
| VS_II0148 | Catalase | 10 | 3.03 | 0.04 | 1.00 | NaN | >100 | >0.23 |
| VS_1054 | Histidine transport ATP-binding protein HisP | 4 | 3.02 | 0.03 | 1.00 | NaN | >100 | >0.23 |
| VS_1129 | Signal transduction histidine kinase | 4 | 2.57 | 0.01 | 1.00 | NaN | >100 | >0.23 |
| VS_II1090 | Phosphomethylpyrimidine kinase | 2 | 2.40 | 0.03 | 1.00 | NaN | >100 | >0.23 |
| VS_2771 | Fructose-1,6-bisphosphatase Fbp | 17 | 2.36 | 0.00 | 1.16 | 0.18 | 8 | 0.101 |
| VS_II1225 | Phosphoenolpyruvate synthase PpsA | 40 | 2.11 | 0.01 | 1.21 | 0.67 | >100 | >0.23 |
| VS_2300 | Arsenate reductase | 3 | 2.09 | 0.02 | 0.97 | 0.97 | >100 | >0.23 |
| VS_II0877 | MoxR-like ATPase | 6 | 2.01 | 0.03 | 1.00 | NaN | >100 | >0.23 |
| **Downregulated proteins** | | | | | | | | | |
| VS_2926 | Ornithine carbamoyltransferase | 17 | 0.49 | 0.04 | 1.17 | 0.76 | >100 | >0.23 |
| VS_2263 | Glutathione-regulated potassium-efflux system protein kefb | 4 | 0.48 | 0.03 | 1.07 | 0.83 | >100 | >0.23 |
| VS_II0953 | Nitrate reductase, large subunit | 3 | 0.45 | 0.04 | 1.00 | NaN | >100 | >0.23 |
| VS_2312 | Arsenate reductase | 4 | 0.45 | 0.04 | 1.00 | NaN | >100 | >0.23 |
| VS_II0935 | ABC transporter substrate-binding protein | 7 | 0.43 | 0.04 | 1.42 | 0.35 | >100 | >0.23 |
| VS_II0219 | Maltose/maltodextrin transporter ATP-binding protein MalK | 23 | 0.42 | 0.00 | 0.47 | 0.10 | >100 | >0.23 |
| VS_1158 | Hypothetical protein VS_1158 | 15 | 0.39 | 0.03 | 2.01 | 0.15 | >100 | >0.23 |
| VS_II0405 | TRAP dicarboxylate transporter subunit DctP | 14 | 0.37 | 0.03 | 0.67 | 0.29 | >100 | >0.23 |
| VS_2935 | Sodium/solute symporter | 3 | 0.23 | 0.04 | 1.00 | NaN | >100 | >0.23 |
| VS_0474 | Glutamate synthase (NADPH) large chain | 64 | 0.19 | 0.00 | 1.02 | 0.39 | >100 | >0.23 |
| VS_1157 | Histidine kinase | 8 | 0.13 | 0.00 | 0.75 | 0.71 | >100 | >0.23 |

*aFDR, false-discovery rate.*
between Vsr217 and fbp mRNA was dependent upon Hfq, we also transformed a MG1655 Δhfq mutant with the same set of plasmids.

The fbp-gfp fusion had a 2-fold reduction of expression in the Δhfq mutant compared to that in the WT strain, but the presence of pPlac-Vsr217 had no effect in the absence of Hfq, indicating that the interaction was Hfq dependent. Altogether, these results indicate that fbp mRNA is submitted to posttranscriptional inhibition by Vsr217 in an Hfq-dependent manner.

**DISCUSSION**

*V. tasmaniensis* LGP32 Vsr217, identified in a genome-wide search for sRNAs (19), is highly induced during oyster infection (18). In this study, we show that vsr217 is a conserved gene in *Vibrio*, located upstream of *malK*, which is inducible by maltose in a MalT-dependent manner. Induction of Vsr217 by maltose could account for its high expression in oysters. Indeed, the latter are rich in glycogen, a precursor of maltose, and the maltose regulon of *V. tasmaniensis* is induced upon oyster colonization (18).

An original feature of Vsr217 is that it is generated from the 5\' UTR of *malK* mRNA as attested by the detection of a vsr217-malK RNA. *malK* is transcribed from two promoters, P1, upstream of vsr217, and P2, located in the intergenic region between vsr217 and malK. P2 is responsible for the transcription of more than half of malK 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. Altogether, these results are in favor of Vsr217 being generated by the
processing of the larger transcript stemming from the P1 promoter, although we cannot exclude that Vsr217, which is much more stable than the malK transcript(s), is generated by termination of transcription at its unique 3' end. However, no Rho-independent terminator was detected to support this hypothesis. In addition, we found that deleting the conserved 13 last nucleotides of vsr217 (called here polyT motif) led to a nearly complete absence of Vsr217 but had no effect on vsr217-malK or malK mRNA levels. These results suggest that the polyT motif is important for Vsr217 stability but not for vsr217-malK processing.

Vsr217 RNA has two functions. In cis, it affects the stability of the malK mRNA, which decreases in the Δvsr217 deletion strain, leading to a reduction of MalK and a decreased ability to use maltose as a carbon source, which is suppressed by the expression in trans of malK but not of vsr217. In addition, Vsr217 acts as a bona fide sRNA to control the expression of fbp, a gene involved in the neoglucogenesis/glycolytic pathway. A decrease of fbp mRNA was observed in the presence of maltose, when Vsr217 is strongly induced, whereas no change was observed in glucose. This decrease was abolished in the ΔpolyT mutant, confirming that it is Vsr217 dependent in the WT strain. Direct interaction between Vsr217 and fbp was further supported by Vsr217-dependent downregulation of a fbp:gfp reporter fusion in E. coli (Fig. 6). In addition, we found that the Vsr217-dependent posttranscriptional downregulation of the fbp:gfp fusion is indeed dependent upon Hfq in E. coli. V. tasmaniensis Hfq has 94% identity and 99% similarity with E. coli Hfq. Accordingly, it is likely that the Vsr217-fbp interaction is dependent upon Hfq in V. tasmaniensis as well.

Interestingly, fbp encodes one of the two enzymes in the glycolytic/gluconeogenesis pathway that catalyze the two irreversible reactions distinguishing neoglucogenesis from glycolysis, the other one being ppsA, encoding phosphoenolpyruvate synthase.
PpsA was also identified in our proteomic approach as increasing in the Δvsr217 mutant, suggesting that it could also be negatively regulated by Vsr217. However, ppsA mRNA was not predicted as a target by CopraRNA. Further studies will be required to determine the potential involvement of Vsr217 in ppsA expression.

In *E. coli*, two major transcriptional regulators have been described to regulate the central carbon metabolism, CRP (catabolite repressor protein) and Cra (catabolite repressor activator, also known as FruR) (30–32). Whereas on neoglucogenic substrates such as acetate, CRP activates key enzymes of glycolysis, Cra downregulates the majority of enzymes in the glycolytic pathway and activates the two gluconeogenesis genes *fbp* and *ppsA*. The action of Cra is dominant over that of CRP (33). Both CRP and Cra have homologues in *V. tasmaniensis*, VS_2855 and VS_II1031, respectively. We propose that Vsr217 contributes to fine-tuning the production of a gluconeogenic enzyme, bringing an additional control to *Vibrio* metabolic regulations (Fig. 7).
adenylate cyclase: cAMP accumulates in the cell, activating CRP, which upregulates glycolysis. Maltose is transformed into glucose-6-phosphate and enters the glycolytic pathway. However, upregulation of glycolysis is counteracted by Cra, which drives the carbon flux toward gluconeogenesis. On the other hand, induction of the maltose regulon leads to a strong increase of Vsr217 that will inhibit the expression of the gluconeogenic enzyme Fbp, thus favoring glycolysis. Hence, in oysters, which are rich in the maltose precursor glycogen, Vsr217, a member of the growing family of trans-acting sRNAs derived from a 5′ UTR, may contribute to the fitness of V. tasmaniensis by optimizing maltose utilization through inhibition of gluconeogenesis.

MATERIALS AND METHODS

**Bacterial strains and plasmids.** 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 (34). The presence of the desired mutations was confirmed by sequencing. Plasmids were constructed by one-step isothermal assembly (Gibson assembly) (35). Their main features are indicated in Table 1, and details of construction are presented in Fig. S2 in the supplemental material. We also constructed a derivative of the cloning vector pXG-10 (26), deleting the fragment coding for the alpha peptide present upstream of the gfp gene which led to a background expression of gfp. The resulting plasmid, pXG-10/Δα was used as a control plasmid to determine the basal intrinsic cellular fluorescence level. Plasmids expressing either Vsr217 or MalK alone, under the control of their native promoters P1 and P2, were constructed by deleting either coding region from a parental plasmid carrying the complete locus P1-Δsr217-P2-ΔmalK (Fig. S2). Primers used for DNA amplification are listed in Table 3.

**Media and bacterial growth.** E. coli strains were cultured at 37°C in Luria-Bertani (LB) broth. V. tasmaniensis LGP32 and its derivative were grown at 20°C in Zobell medium (4 g/liter peptone, 1 g/liter yeast extract, 0.1 g/liter ferric phosphate, and 30 g/liter sea salt) or minimal medium (3 g/liter KH₂PO₄, 7 g/liter K₂HPO₄, 2 g/liter (NH₄)₂SO₄, 0.5 × 10⁻³ M g/liter FeSO₄, and 30 g/liter NaCl) supplemented with 2 g/liter o-glucose or o-maltose, where indicated. Antibiotics were used as follows: 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 0.5 mM.

To measure growth of LGP32, bacteria (3 replicate cultures in each case) were cultured overnight in minimal medium supplemented with 2 g/liter o-glucose or o-maltose. If needed, Cm was added. Overnight cultures were diluted to an optical density at 600 nm (OD₆₀₀) of ~0.05 in the same medium, and growth was monitored in a 96-well plate, by measuring cell density at OD₆₀₀ at regular time intervals in a CLARIOstar (BMG Labtech, France) microplate reader.

**RT-PCR and Northern blotting.** Overnight cultured bacteria were diluted to an OD₆₀₀ of ~0.05 in minimal medium, supplemented by glucose or maltose where indicated, except for the results presented in Fig. 2, where cells were grown in Zobell medium, since the malT mutant does not grow in minimal medium plus maltose. Cells were grown with continuous shaking at 180 rpm at 20°C. When the OD₆₀₀ reached ~0.4, bacteria were collected by centrifugation and resuspended in TR 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. Ten micrograms (5 μg in the case shown in Fig. 4B) purified RNA was used for Northern blotting as described previously (19). Oligonucleotide probes (Table 3) complementary to the gene to be assayed were labeled at their 3′ ends using terminal transferase (Fermentas, USA) and [α-³²P]dCTP, according to the manufacturer’s instructions. Transfer-messenger RNA (tmRNA) was used as a loading control. Membranes were scanned using an Amersham Typhoon biomolecular imager (GE Healthcare) in the phosphorimager mode. Transcript signals were quantified by ImageJ (36) 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 Vs217-RT-F and MalK-RT-R (Table 3). 5′ and 3′ rapid amplification of cDNA ends. Overnight cultures of LGP32 WT and Δvsr217 strains were diluted to an OD₆₀₀ of ~0.05 and grown in minimal medium supplemented with 2 g/liter o-maltose. Bacteria were collected by centrifugation when the OD₆₀₀ reached ~0.4 and were resuspended in TRI reagent followed by RNA extraction, DNase treatment, and RNA purification. 5′ and 3′ ends of Vs217 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 3. In each case, the subsequent PCR products were cloned in the pJET1.2 vector (Thermo Fisher Scientific). Twenty plasmids were prepared from randomly chosen colonies, and inserts were sequenced using oligonucleotides pJET1.2-F/R (Table 3).

**RNA stability measurement.** LGP32 WT, Δvsr217, and ΔpolyT strains were grown overnight in minimal medium supplemented with 2 g/liter o-maltose at 20°C. Bacteria were diluted to an OD₆₀₀ of ~0.05 and grown in the same medium until an OD₆₀₀ of ~0.4 and rifampicin was added to a final concentration of 500 μg/ml. Bacteria were collected for RNA extraction at 0, 0.5, 1.0, 2.0, and 3.0 min after the addition of rifampicin. Northern blot analysis was performed to determine the amount of Vs217 and malK transcripts.
### TABLE 3 Oligonucleotides used in this study

| Name | Sequence (5'→3') | Target/goal |
|------|------------------|-------------|
| **Northern blot probes** | | |
| NB-vs-MalK | CATACCTACACACACGTGGTTGAGCC | malK |
| NB-vs-Vsr217 | CCCGTGATCTCAATCACAGATTTGCTCGA | Vsr217 |
| NB-vs-Fbp | ATCTCAGGTTAACATTTTTCGAG | fbp |
| NB-vs-tmRNA | AGCCACGCTACTGTTGGGTTTGTAC | tmRNA |
| **RT-PCR** | | |
| Vsr217-RT-F | ACCACGAATTGTTAGTG | Vsr217 |
| MalK-RT-R | CAAGCGACGACATGCTCTAC | malK |
| **5' and 3' RACE** | | |
| Oligo(dT)-anchor primer | GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTT | RACE product cloning |
| PCR Anchor primer | GACCACGCGTATCGATGTCGAC | RACE product cloning |
| 5' MalK-GSP1 | GAATACCATACCTACACCACGT | MalK |
| 5' MalK-GSP2 | ACGTAAATCTTCTAAGACCTGCGAT | MalK |
| 5' MalK-GSP3 | CCACTAATAGAAGAATTCGTCACAC | MalK |
| 3' MalK-GSP1 | TGGCGACATTGAAGCTGATG | MalK |
| 3' MalK-GSP2 | GGTATTCCTGCACACCGTG | MalK |
| 3' Vsr217-GSP1 | ACCACGAATTGTTAGTG | Vsr217 |
| 3' Vsr217-GSP2 | CTGCCCGCTGTTGTCTTTAT | Vsr217 |
| **vsr217- and malK-expressing plasmids** | | |
| pGBE12-F | CACTGGCCGTCGTTTTTAACGTCG | pGBE12 |
| pGBE12-R | CAAGCTTGCATGCCTGCAGGTCGAC | pGBE12 |
| Vsr217-malK-F | aggcatgcaagcttgCCCTTTATCTACTCCTCCCC | P1-vsVsr217-P2-malK plasmid |
| Vsr217-malK-R | aaacgacggccagtgAACAGACAAAGCGCGATC | P1-vsVsr217-P2-malK plasmid |
| del vsr217-F | TTACTCACTCAATACTTACCAATTCCTACAGTTACTGCTG | Deleting vsr217 to get pP1-P2-MalK |
| del vsr217-R | GGTAAGTATTGAGTGAGTAAGAATGCAGTTTCGGTGAATT | Deleting vsr217 to get pP1-P2-MalK |
| del malK-F | TCGGTTCGTCTTTTTAGAATATAACTTGCCCGTATCGAAT | Deleting malK to get pP1-Vsr217 |
| del malK-R | TTCTAAAAAGACGAACCGAATTGAGTGAGTAAGAAAAAAGTGGGTGGG | Deleting malK to get pP1-Vsr217 |
| **Mutagenesis** | | |
| pSW7848-F | GTCTGATTCGTTACCAATTATGACAAC | Vector amplification |
| pSW7848-R | GAATTCGATATCAAGCTTATCGATAC | Vector amplification |
| Vsr217-up-F | aagcttgatatcgaattcTCTTCCCAAGACTTAGGTGGGTTAGGGACAAG | Upstream of vsr217 |
| Vsr217-up-R | aaagcagcagctgcACGAGGACAAAGGCGGATC | Upstream of vsr217 |
| Vsr217-down-F | ctgcattcTACTCACTTCAATACCTAACATACGCTAATGCTGAC | Downstream of vsr217 |
| Vsr217-down-R | ttcgtaagactgcagccCACAGAAGAAGGTTTTAGGAGATAGTTG | Downstream of vsr217 |
| malT-up-F | aagcttgatatcgaattcTAATTCGTAGTGAAGACCGTAGCC | Upstream of malT |
| malT-up-R | cgaagctgCTATTTTGCCTGGTATGTTTT | Upstream of malT |
| malT-down-F | cggagaactgCAAGTGTTAGTTCGCG | Downstream of malT |
| malT-down-R | ttggaagactgcagcagcAGTTGTGAAAGCGAAG | Downstream of malT |
| polyT up-F | ataagcttgatatcgaattcAGACTTAGGTGGGTTAGGGACAAG | Upstream of last 13 nt of vsr217 |
| polyT up-R | ggttgaagactgcagcagcAGTTGTGAAAGCGAAG | Upstream of last 13 nt of vsr217 |
| polyT down-F | ttcaagctccTACTCACTCAATACCTAACCAATTC | Downstream of last 13 nt of vsr217 |
| polyT down-R | taattggtaacgaatcagacCAATCGTCGCATCACCTG | Downstream of last 13 nt of vsr217 |
| **sRNA interaction system** | | |
| pZI2-F | CTAGAGGCATCAAATAAAACGAAAGGCTCA | pZI2MCS |
| pZI2-R | GGAAGTCCTGCTGCCACCTAGTAC | pZI2MCS |
| Vsr217-F | ttaagcggaggaaggtacctCCCTTCTACTCTCCTCCC | Vsr217 |
| Vsr217-R | gttttattctgctctagAAGAAGACAGACAAAGGCGGATC | Vsr217 |
| pXG-10_fwd | GCTAGCAAAGGAGAAGAACTTTTC | Vector amplification |
| pXG-10_rev | GTGCTCAGTATCTCTACAT | Vector amplification |
| pZE-CAT | TGGGATATATCAACGGTGGT | Vector amplification |
| JV0-155 | CGATGCTACGGTATGATA | Vector amplification |
| Vibtas_FBP_UTR_F | atagagatactgagcacATTACATTAAAAAAGATCTAACCTTAG | fbp 5’ UTR (−125, +3) |
| Vibtas_FBP_UTR_R_bis | aagcttctctctgCTACGACTGATATTTCCTTTAACCTTCTC | fbp 5’ UTR (−125, +3) |
| Ptet-R-2 | GTGCTCAGTATCTCTACTTGATA | Deleting the alpha peptide encoding fragment from pXG-10 |
| GFP-F | GCTAGCAAAGGAGAAGAATTTTTC | Deleting the alpha peptide encoding fragment from pXG-10 |

*Non-target bases complementary to the cloning vector are indicated by lowercase letters.
GFP fluorescence measurement. To monitor green fluorescent protein (GFP) fluorescence, strains to be assayed (derived from E. coli MG1655) were grown over-night in LB supplemented with Cm and Amp. Five microliters of the overnight culture was spotted onto LB agar supplemented with Cm, Amp, and IPTG where indicated, and the plates were incubated at 37°C. Plates were then scanned with an Amersham Typhoon model 5 biomolecular imager (GE Healthcare), using the fluorescence mode (excitation [Exc], 488 nm; emission [Em], 525 nm; filter Cy2 525BP20). Signal intensity of the bacterial spots was determined using ImageJ and the formula (integrated density of the spot – [spot area × mean background signal]), where the background signal was the signal obtained for cells containing the non-GFP-expressing plasmid, to remove the intrinsic fluorescence of bacterial cells. Significance of the relative differences was determined by a Student’s unpaired t test, with four biological replicates.

Proteomics. *V. tasmaniensis* LQP32 and *vsr217* strains were grown in triplicates in Zobell with glucose or maltose until an OD_{600} of ~0.4 (12 samples in total). After centrifugation of 2 ml, cells were washed in Tris/EDTA (pH 7.5) plus 20% sucrose and then resuspended in NuPAGE LDS buffer (Thermo Fisher Scientific) to a final concentration equivalent to a OD_{600} of 20. Samples were heated at 95°C for 10 min before loading 10 µl of each sample on a 4% to 12% polyacrylamide gradient gel (Invitrogen). After a 5-min migration to let the samples enter the gels, they were stained in Coomassie blue and then destained.

For tandem mass spectrometry (MS/MS), proteins were subjected to an in-gel enzymatic digestion with trypsin. Nanoscale liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS-MS; Triple-TOF) was carried out (gradient of 120 min), and the peptides were identified by Mascot and Andromeda and quantified by MaxQuant. Statistical analyses were carried out using Perseus (37). P values were calculated using a Welch’s t test.

Bioinformatic predictions. Promoters were predicted using the BPROM software, and Rho-independent terminators were predicted using the FindTerm software (38). Vibrio molfk upstream sequences were aligned using Muscle at the EBI (39). Putative *Vsr217* targets were identified using CopraRNA, and interaction between *Vsr217* and the *fbp* mRNA upstream region was predicted using IntaRNA (23).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**FIG S1**, PDF file, 0.1 MB.

**FIG S2**, PDF file, 0.1 MB.

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Luo et al.