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A novel sRNA *srvg17985* identified in *Vibrio alginolyticus* involving into metabolism and stress response

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ABSTRACT

Vibrio alginolyticus is an opportunistic pathogen that is a threat to the aquaculture industry. Evidence has revealed critical roles for small RNAs (sRNAs) in bacterial physiology and pathology by modulating gene expression post transcription. However, little information about sRNA-mediated regulation in *V. alginolyticus* is available. We experimentally verified the existence and characterized the function of sRNA *srvg17985* in *V. alginolyticus* ZJ-T. We identified a 179 nt and growth-phase-dependent transcript with a σ70 promoter and a ρ-independent terminator. The transcript consisted of five stem-loops and was conserved in *Vibrio* spp. Phenotype microarray assays showed that deletion of *srvg17985* led to less use of Gly-Glu as a carbon source but a gain in ability to use L-phenylalanine as a nitrogen source. *Srvg17985* regulated the osmotic stress response with stronger tolerance to NaCl but weaker tolerance to urea. In addition, *srvg17985* inhibited the deamination of L-serine at pH 9.5 and promoted the hydrolysis of X-beta-D-glucuronide, thus affecting the pH stress response. Bioinformatics by IntaRNA and TargetRNA2 identified 45 common target mRNAs, some of which probably contributed to the observed phenotypes. These results indicated that *srvg17985* regulated environmental adaptation. The results provide valuable information for in-depth studies of sRNA-mediated regulation mechanisms of the complex physiological processes of *V alginolyticus* and provide new targets for antibacterial therapeutics or attenuated vaccines for *Vibrio* spp.

1. Introduction

Bacteria small RNAs (sRNAs) are generally trans-encoded regulatory RNAs of 50–500 nt (Wagner and Romby, 2015). They are transcribed from intergenic regions but not translated and are always conserved among homologs (Storz et al., 2011). The sRNAs typically bind mRNAs through short sequence complementarities and change mRNA stability and translation (Wagner and Romby, 2015). One sRNA usually regulates multiple mRNAs and one mRNA can be regulated by more than one sRNA, thus forming regulatory networks in response to the changing environment (Melamed et al., 2016). A large number of sRNAs have been identified and characterized in bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Vibrio cholerae* and *Vibrio harveyi* (Bak et al., 2015; Pérez-Reytor et al., 2017; Liu et al., 2018; Lu et al., 2018). SRNAs are reported to control a variety of cellular processes including metabolism, stress response, surface composition, and virulence (Kang et al., 2014; Papenfort and Vogel, 2014).

We are interested in the regulatory sRNAome of *Vibrio alginolyticus*. *V. alginolyticus* is widely distributed in estuaries and oceans and poses a great threat to the aquaculture industry and humans by causing serious infections (Deng et al., 2016a). This bacterium has evolved a variety of infection mechanisms, including phase variation, biofilm information, and quorum sensing to adapt to rapidly changing environments including changing pH, salinity, and nutrition, which allow survival in hosts (Ye et al., 2008; He et al., 2011). Since sRNAs represent a new level of post-transcriptional regulation, it is not surprising that these molecules are important in the environmental adaptation and viability

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of *V. alginolyticus*. Silveira et al. (2010) identified, *in silico*, 9 trans-encoded regulatory RNAs in *V. alginolyticus* 40B: one *rheB*, five *qrr*, one *rybB*, one *spot42* and one *gcvB*. Huang et al. (2015) reported three sRNAs involved in adhesion, chemotaxis and motility in *V. alginolyticus*. In a previous study, we comprehensively predicted *V. alginolyticus* sRNAs using an RNA-seq method (Deng et al., 2016a) and identified a novel sRNA that modulates metabolism and stress response in *V. alginolyticus* (Deng et al., 2018). However, more evidence is needed about sRNA regulation that allows *V. alginolyticus* to adapt to changing environments.

Based on our previous study (Deng et al., 2016a), we confirmed the existence and precise transcript of a new sRNA, *srvg17985*, in *V. alginolyticus*. Its sequence features were annotated. Additionally, phenotypic microarray (PM) techniques were used to study the function of the sRNA in the metabolism of carbon and nitrogen sources and the response to osmotic and pH stress. Phenotypic regulation was linked to genetic regulation by *in silico* target prediction. These results will help to understand the effects of sRNAs in the environmental adaptation of *V. alginolyticus* and provide new insights into its pathogenesis. The results will assist in providing new targets for developing antibacterial therapeutics or attenuated vaccines against *Vibrio* spp.

2. Materials and methods

2.1. Growth of bacterial strains

V. alginolyticus ZJ-T used in this study was the translucent/smooth variant of wild strain ZJ-51 which was isolated from diseased *Epinephelus coioides* off the Southern China coast and virulence to *E. coioides* (Chen et al., 2009). Here, *V. alginolyticus* strains were cultured in LBS [Luria-Bertani (LB) with 2% additional NaCl], Zobell (BD, USA) or TCBS (BD, USA) medium at 30 °C. *E. coli* Π3813 and GEB883 were used for gene deletions and were cultured in LB (BD, USA) medium supplemented with 0.3 mM 2'-deoxythymidine and 0.3 mM 2,6-diaminopimelic acid at 37 °C. If needed, 5 μg/mL chloramphenicol was added to media for *V. alginolyticus* strains and 20 μg/mL for *E. coli* strains. Bacterial strains and plasmids used in this study are in Table 1. Nucleotide sequences of primers or probes used in this study are in Table 2.

2.2. Northern blots

Bacterial cells were collected at indicated time points along the growth curve of *V. alginolyticus* ZJ-T in LBS and Zobell medium for total RNA extraction. Extraction used TRI Reagent (Sigma-Aldrich, USA) and 10 μg RNA from each sample was used for northern blots as described previously (Deng et al., 2018). Probes NB-*srvg17985* and NB-5.0S RNA (Table 2) were labeled at the 3' end using terminal transferase (Fermentas, USA) and [α-32p] dCTP. RiboRuler Low Range RNA Ladder (Fermentas, USA) was used as a size marker for comparison and estimation of transcript sizes. Transcripts were quantified by Image J (Schindelin et al., 2015). Expression of *srvg17985* was normalized to 5.0S RNA.

2.3. 5' and 3' rapid-amplification of cDNA ends

RNAprep Pure Cell/Bacteria Kits (TIANGEN, China) were used to isolate total RNA from bacterial cells in LBS medium at OD_{600} = 0.8. SMARTer® rapid-amplification of cDNA ends (RACE) 5'/3' Kits (Clontech, USA) were used to map transcript 5' and 3' ends. PCR products were cloned into pUC19 (Clontech, USA). At least 12 resulting clones were sequenced to determine *srvg17985* ends.

2.4. Transcript characterization and phylogenetic analysis

The Rfam database was used to assess transcript novelty using Blast methods (Gardner et al., 2008). By employing BPROM (Solovyev et al., 2010) and ARNold (Naville et al., 2011), promoters and terminators were detected within 200 nt upstream and downstream of the transcript. The MFOLD (Zuker, 2003) program was used to predict secondary structure basing on folding energy. Synteny and sequence conservation were analyzed by Blast analysis against the National Center for Biotechnology Information (NCBI) database using the sequences of the promoter, transcript and terminator. Multiple sequence alignment was by ClustalX (version 1.8) (Thompson et al., 2003). Basing on aligned sequences, MEGA6.0 software was used to construct phylogenetic trees using the neighbor-joining method with the setting bootstrapped 1000 times (Tamura et al., 2013).

2.5. In silico target gene predication

IntaRNA (Siqueira et al., 2016) and TargetRNA2 (Kery et al., 2014) were used to predict the target genes of *srvg17985* as described previously (Deng et al., 2018). The hybridization was screened between the sRNA transcript sequence and 150 nt sequence flanking the start codon of each annotated gene in the genome of *V. alginolyticus* ZJ-T. The corresponding $p < 0.05$ of both programs, energy < -13 kcal/mol with IntaRNA, and synonym energy < -8 kcal/mol with TargetRNA2 were taken as the threshold to consider an interaction as positive. Common predictions were parsed based on their KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway and GO (Gene Ontology) annotation confirming target candidates.

2.6. Gene deletion

The sRNA knockout mutant was constructed as described previously (Deng et al., 2018). Recombinant plasmid pSW7848-Δ*srvg17985* with two flanking fragments of *srvg17985* which were amplified with the primer pairs of *srvg17985*-UP-F and -R and *srvg17985*-DOWN-F and -R (Table 2) respectively, and linearized pSW7848 which was amplified with pirmer pair of pSW7848-F and -R (Table 2) were obtained by isothermal assembly using ClonExpress MultiS One Step Cloning Kits

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Sources
V. alginolyticus		
ZJ-T	Ap^r (ampicillin resistant), translucent/smooth variant of wild strain ZJ-51(Huang et al., 2017); isolated from diseased *Epinephelus coioides* off the Southern China coast	(Chen et al., 2009)
ZJ-T-Δ*srvg17985*	Apr; ZJ-T carrying an deletion of *srvg17985*	This study
E. coli		
Π3813	Emr^r, Tc^r, *lacIQ, thi1, supE44, endA1, recA1, hsdR17, gyrA462, zei298::tn10[Tc], ΔthyA:: (erm-pir116)*; the intermediate host of suicide vector pSW7848	(Le Roux et al., 2007)
GEB883	Ery^r, Tet^r, WT *E.coli* K12 *ΔdapA::erm pir RP4-2 ΔrecA gyrA462, zei298::Tn10*; donor strain for conjugation	(Nguyen et al., 2018)
Plasmids		
pSW7848	Cmr; suicide vector with an R6K origin, requiring the Pir protein for its replication, and the *ccdB* toxin gene	(Val et al., 2012)
pSW7848-Δ*srvg17985*	Cmr; pSW848 containing the mutant allele of Δ*srvg17985*	This study

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

Name	Sequence (5'–3')	Goal
NB-*srvg17985*	ACGAGGACTTACCGTAGGGTTTGAA	Northern blot
NB-5.0S RNA	CCCCACACTACCATCGGCGCTATT	
RACE-UPML	TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	Race
RACE-UPMS	CTAATACGACTCACTATAGGGC	
race-*srvg17985*-F	GATTACGCCAAGCTTTGCTTCAGAAGGAGGGATGTGAGGT	
race-*srvg17985*-R	GATTACGCCAAGCTTGCGCACATGTTCGTGCGCCT	
pSW7848-F	GTCTGATTCGTTACCAATTATGACAAC	Mutagenesis
pSW7848-R	GAATTCGATATCAAGCTTATCGATAC	
srvg17985-UP-F	aagcttgatatcgaattcCACCACCAGTTAGTTGGG	
srvg17985-UP-R	caatcgtgaAGCTAAATTGCATCTTTATTG	
srvg17985-DOWN-F	caatttagctTCACGATTGAGTAACGAGG	
*srvg17985*DOWN-R	ttggtaacgaatcagacCTAACCAAAGCAAATCAAATTG	
Del-check-pSW7848-F	TCACTGTCCCTTATTCGCACC	
Del-check-pSW7848-R	CTGCTTTTGAGCACTACCCG	
Δ*srvg17985*-check-F	GACCCTCGAGAGACAAACTGGTTA	
Δ*srvg17985*-check-R	GCACTGCTCTAGTTGGTTGACATC	
RT-BAU10_17985-UP-F	CACGCCTCCCAGTAACGAAC	RT-PCR
RT-BAU10_17985-UP-R	CGCATTGTTTGGCGTGTTTG	
RT-BAU10_17990-DOWN-F	CTGGTATGGTTGCGAAGTGC	
RT-BAU10_17990-DOWN-R	ACGCTGCCACTCGTATTCAG	
RT-*srvg17985*-F	CAGAAGGAGGGATGTGAGGT	
RT-*srvg17985*-R	ATCGTGAGTGTGCGAATCGG	
gyrA-F	TCGAAGAAGGTGAGCGCATT	
gyrA-R	TCACAGCGATTAGGCCGTTT	
uvrA-F	GAAGGAAATCAACGACCGCC	
uvrA-R	GGCCAATCGACGGTTCATCT	
recA-F	TCTTCATCAACCAAATCCGTA	
recA-R	GCTGCAATCTTATTCTTAACAACTT	

(Vazyme, China) and transferred into *E. coli* Π3813 and GEB883 successively (Table 1). Plasmid pSW7848-Δ*srvg17985* was transferred to *V. alginolyticus* ZJ-T by conjugation. Then TCBS with 0.2% D-glucose and 5 μg ml^{-1} chloramphenicol (Cm) was used to select transconjugants that have undergone first allelic exchange. Subsequently, TCBS with 0.2% L-arabinose was used to select the transconjugants that have undergone plasmid excision and second allelic exchange. The expression of the *ccdB* gene (Loris et al., 1999) carried by the pSW7848 plasmid was induced by L-arabinose in the medium. After two allelic exchanges, the mutation was confirmed by sequencing with the primers of Δ*srvg17985*-check-F and -R (Table 2).

2.7. RT-PCR

RT-PCR was used to test polar effects of the deletion on flanking genes using primers in Table 2. RNAiso Plus (Takara, Japan) was used to isolate total RNA from bacterial cells ZJ-T and ZJ-T-Δ*srvg17985* in LBS medium at OD_{600} = 0.5. Reverse transcription was by PrimeScript™ RT reagent kits with gDNA Eraser (Takara, Japan). PCR amplification used SYBR Premix Ex Taq™ II (Takara, Japan). Experiments were conducted in triplicate. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with genes *recA*, *uvrA*, and *gyrA* as controls and normalizing to the value for wild type ZJ-T. Statistical significance was determined with independent *t*-test.

2.8. Measurement of bacterial growth

Single clone of the wild type ZJ-T and the mutant ZJ-T-Δ*srvg17985* were grown individually overnight in LBS medium at 30 °C, 200 rpm. The overnight cultures were diluted with fresh LBS (1:500) and incubated at 30 °C, 200 rpm. The OD_{600} was measured at regular time intervals using a spectrophotometer (Thermo Fisher Scientific). The experiment was done triplicates and the result was shown with graphs. A one-way analysis of covariance (ANCOVA) was conducted to analyze the effect of *srvg17985* deletion on OD_{600} with time as a covariate.

2.9. PM assays

Four 96-well PM panels (Supplementary Data 1: carbon sources metabolic panel PM01, nitrogen sources metabolic panel PM03, osmotic stress response panel PM09, and pH stress response panel PM10) were used to investigate the function of *srvg17985* (Bochner et al., 2001). Tests were performed essentially as described by Deng et al. (2018). For PM01, cells from LBS agar plates were transferred and suspended into 1.0 × IF-0 containing 1 × Dye Mix A and 3% salinity to achieve 85% T (transmittance) cell suspension. The cell suspension was added into PM01 panel with 100 μl per well. For PM03, appropriate sodium succinate was added into the 85% T cell suspension prepared for PM01 with a finally concentration of 2 mM. Then, each well of the PM03 plate was inoculated with 100 μl of the prepared cell suspension. For PM09 and PM10 experiments, the 85% T cell suspension prepared for PM01 was diluted 200 times with the inoculating fluid of 1.0 × IF-10 containing 1 × Dye Mix A and 3% salinity. Then, the cell suspension was added into PM09 and PM10 panels with 100 μl per well. Panels were inoculated at 30 °C and OD_{590} (Blumenstein et al., 2015) was measured during growth using a Multiskan Ascent plate reader (Thermo Fisher Scientific, USA). Experiments were performed in triplicate. ANCOVA was conducted to analyze the effect of *srvg17985* deletion on OD_{590} with time as a covariate. Significantly different phenotypes were described with graphs and in a heat map creating using the maximum difference in OD_{590}.

3. Results

3.1. Locus feature and srvg17985 transcript

Srvg17985 with a size of 188 nt was shown in an Artemis window flanked by BAU10_17985, the H(+)/Cl(-) exchange transporter ClcA-encoding gene, and BAU10_17990, a hypothetical protein-encoding gene (Fig. 1A). A band was detected on northern blots with a size around 180 nt (Fig. 1B–C). During bacterial growth, expression of *srvg17985* was induced both in LBS and Zobell medium (Fig. 1B–C).

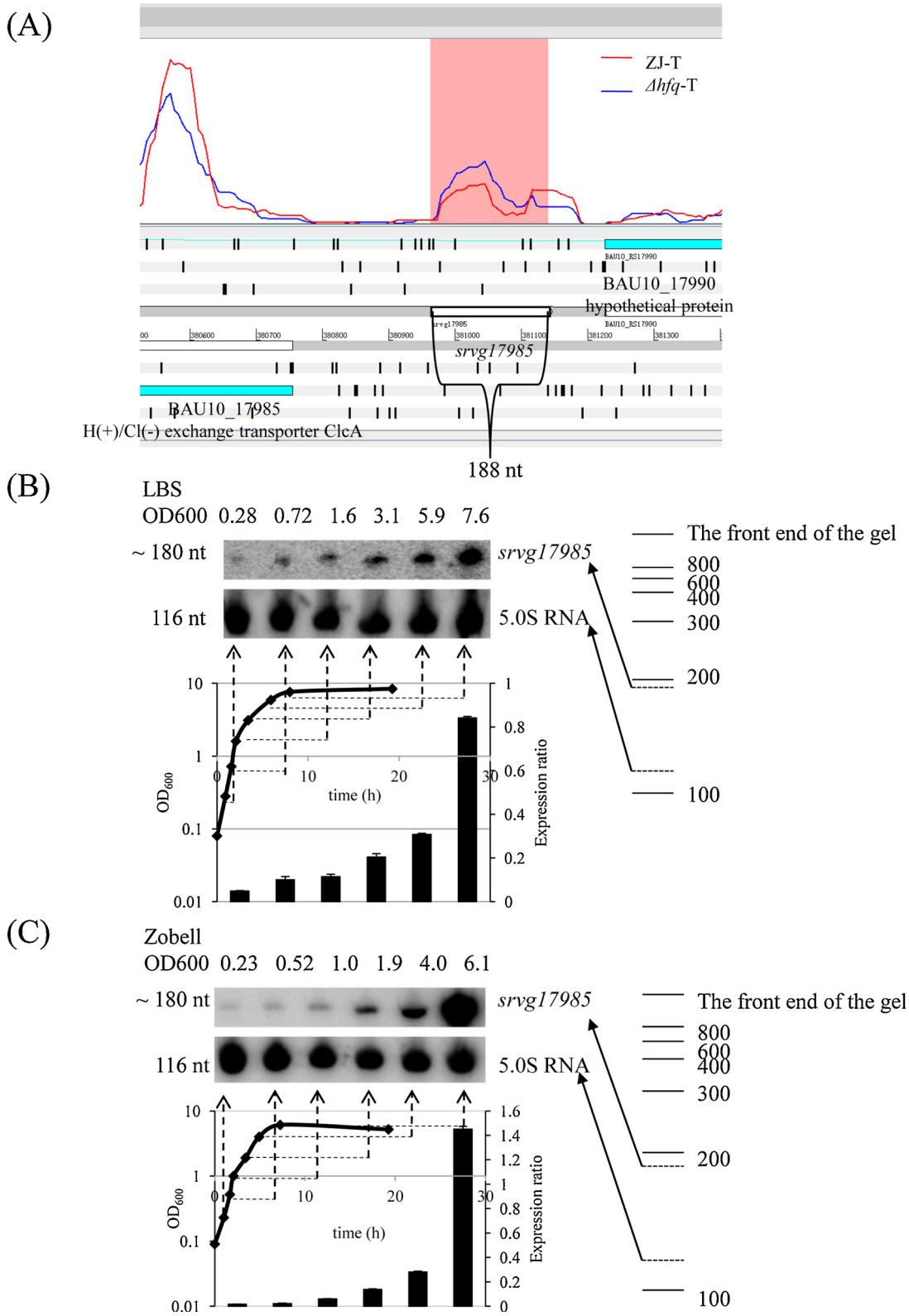

Fig. 1. Locus features and expression of *srvg17985*. (A) *srvg17985* was flanked by BAU10_17985 and BAU10_17990, shown in an Artemis window. (B) Northern blots of *srvg17985* with bacterial growth in LBS medium and expression normalized to the control, 5.0S RNA. (C) Northern blots of *srvg17985* with bacterial growth in Zobell medium with expression normalized to the control, 5.0S RNA.

3.2. Secondary structure, transcript features and phylogenetic analysis

A single band was detected by 5′ RACE and 3′ RACE (Fig. S1). Sequencing showed that the most abundant transcript was 179 nt. Novelty assessment revealed that *srvg17985* was first found. A σ70 promoter was detected at the 5′ end with a -35 box, a -10 box, and two transcriptional regulatory factors, IHF-box and FNR-box (Fig. 2A). A ρ-independent terminator was detected at the 3′ end (Fig. 2B). In

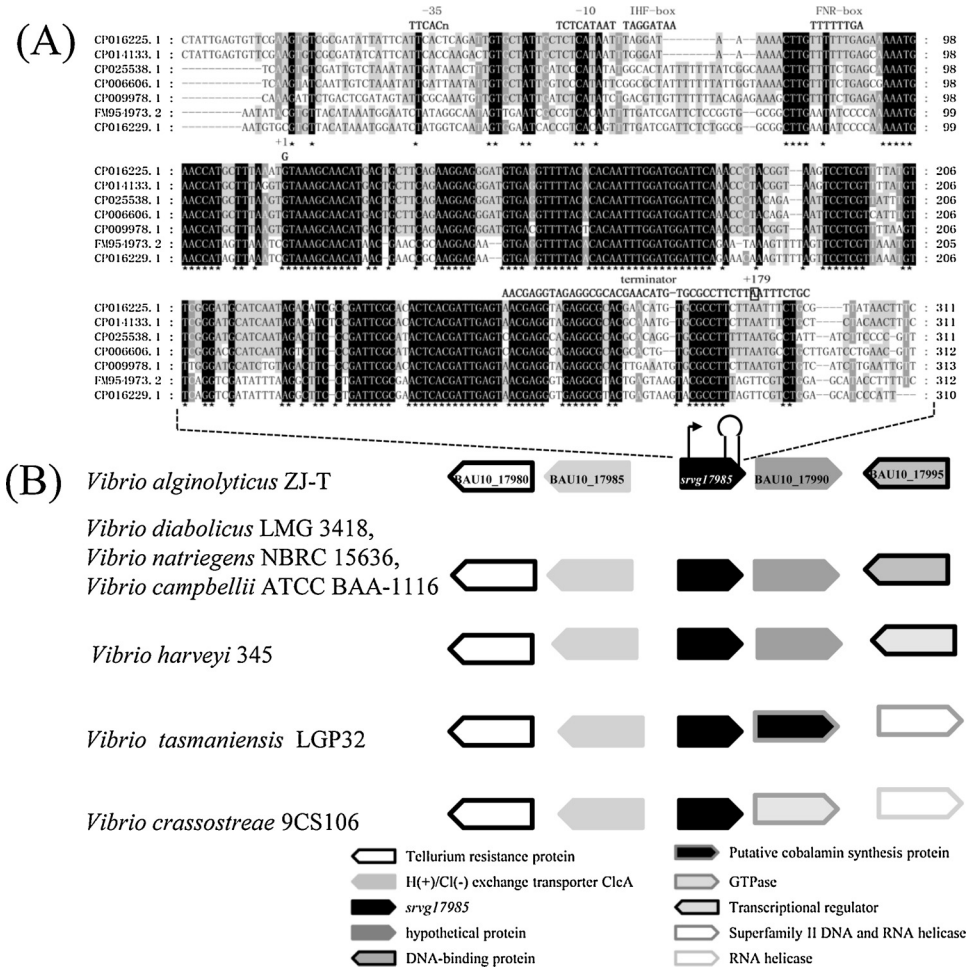

Fig. 2. Transcript features and conservation analysis of *srvg17985*. (A) Multisequence alignment of possible *srvg17985*-analogous genes in available genome sequences of *Vibrio* spp. Asterisks: absolutely conserved in all sequences. Features: transcriptional start site (+1), transcriptional stop site (+179), -35 box, -10 box, IHF-box and FNR-box in a σ70 promoter, and ρ-independent transcriptional terminator. (B) Diagrammatic representation of *srvg17985* synteny conservation among *V. alginolyticus* ZJ-T and other *Vibrio* spp. Organization and orientation of annotated genes and putative sRNA gene are indicated.

sequence conservation analysis, the sequence of the transcript and terminator were highly homologous among all seven *Vibrio* spp., while the sequence of the promoter showed low similarity (Fig. 2A). The -35 box, -10 box, and FNR-box were conserved in *V. alginolyticus* and *Vibrio diabolicus*, and the IHF-box was specific in *V. alginolyticus* (Fig. 2A). In synteny conservation analysis, a similar organization of the flanking gene locus was seen among the less-distant spp. *V. alginolyticus*, *V. diabolicus*, *Vibrio natriegens*, *Vibrio campbellii*, and *V. harveyi* (Fig. 2B). The right flanking locus appeared to be a region of chromosomal rearrangement in the more distant spp. *Vibrio tasmaniensis* and *Vibrio crassostreae* (Fig. 2B). *Srvg17985* formed a five stem-loop structure with a *dG* of -58.38 kJ (Fig. 3A) and was conserved among *Vibrio* spp (Fig. 3B).

3.3. Common targets

We identified 45 common targets by IntaRNA and target RNA2 with *p*_value < 0.05 of both programs, and energy < −13 kcal/mol with IntaRNA and synonym energy < −8 kcal/mol with TargetRNA2 and TargetRNA2 (Siqueira et al., 2016; Kery et al., 2014). According to the KEGG orthology on level 1–3, they were involved into a series of cellular processes, including metabolism (BAU10_07995, BAU10_08780, BAU10_09100, BAU10_22375, BAU10_02050, and BAU10_06225), transporters (BAU10_17155, BAU10_17180, BAU10_23950, BAU10_23790, BAU10_17800, BAU10_20040), quorum sensing (BAU10_18875), two-component system (BAU10_08620), and biofilm information (BAU10_21995) (Supplementary Data 2).

3.4. Knockout mutant

A total of 141 bp from the 5' end of the *srvg17985* transcript was deleted from *V. alginolyticus* ZJ-T (Fig. 4A). RT-PCR was performed to verify the polar effect of the deletion on the neighboring genes. The results showed that the expression of *srvg17985* upstream and downstream genes was not altered significantly in ZJ-T-Δ*srvg17985*, while the expression of *srvg17985* was not detected in ZJ-T-Δ*srvg17985* which meant that the *srvg17985* was successfully deleted and there was no polar effect happened (Fig. 4B).

3.5. Phenotypic characterization

ANCOVA showed that there was no significant growth difference between the wild type and the mutant ZJ-T-Δ*srvg17985* in LBS medium ($F = 0.008$, $P = 0.931$) (Fig. S2). PM assay results are in Figs. S3–S6. Phenotypes gained and lost are in Table 3 and Fig. 5, and the original data are in Supplementary Data 3. The mutant had weaker utilization of Gly-Glu (PM01-G01) as a carbon source and lower viability during stationary phase (Fig. 5A-a). No difference was observed for metabolism of L-glutamic acid (PM01-B12), Gly-Asp (PM01-F01), Ala-Gly (PM01-G06), or Gly-Pro (PM01-H01) between the wild type and mutant (Fig. S3). The mutant gained the ability to use L-phenylalanine as nitrogen source (PM03-B08) (Fig. 5A-b). Though both strains tolerated as

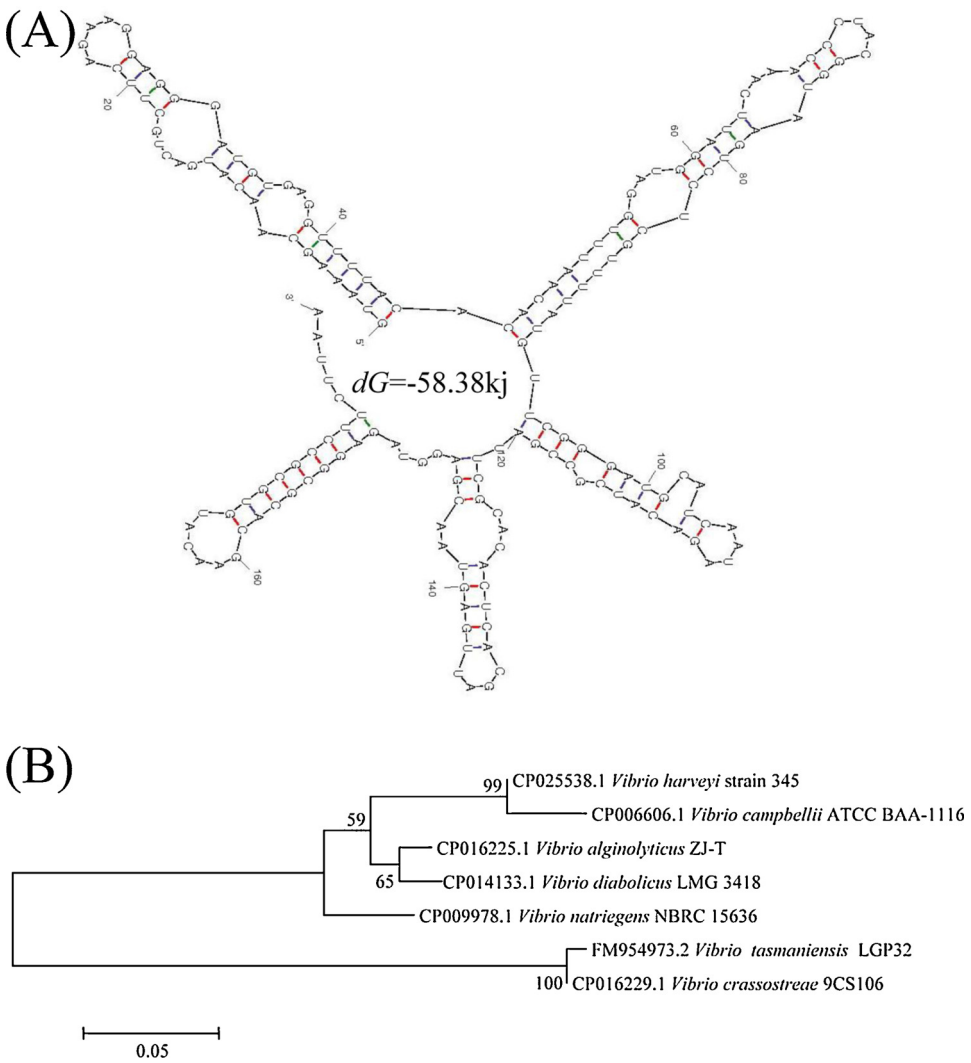

Fig. 3. Secondary structure and phylogenetic analysis of *srvg17985*. (A) *Srvg17985* consisted of five stem-loops with a *dG* of −58.38 kJ in *V. alginolyticus* ZJ-T. (B) Phylogenetic tree was constructed according to multisequence alignment results using MEGA6.0 software. Relative bootstrap (%, Bootstrap values ÷ 1000 × 100) are on branch points.

much as 10% NaCl, the mutant showed a higher growth rate and viability, especially with 9% and 10% NaCl (PM09-A11, PM09-A12) (Figs. S5A1–A12, 5A-c, A-d). With increased urea concentration, bacterial growth decreased, stopping at a concentration of 6% (Fig. S5E7-E12). The mutant strain showed a weaker tolerance to urea, especially 3% and 4% (PM09-E08, PM09-E09) and had lower growth at stationary phase (Fig. 5A-e, A-f). The mutant gained growth when exposed to pH 9.5 with L-serine (PM10-F04) and showed a weaker tolerance to X-beta-D-glucuronide and lower growth at stationary phase (PM10-H07) (Fig. 5A-g, A-h).

4. Discussion

Numerous studies show that sRNAs are critical for regulating bacterial genetic variation, growth and reproduction, and pathogenesis (Wagner and Romby, 2015; Klein and Raina, 2017; Fröhlich and Gottesman, 2018). Therefore, identifying sRNAs and studying their regulatory function is important for understanding the environmental adaptability of bacteria. We identified a novel sRNA, *srvg17985*, which was predicted by using an RNA-seq strategy (Deng et al., 2016a). We comprehensively characterized the sRNA function using PM technology. Additionally, phenotypic regulation was linked to genetic regulation by target prediction.

Size was identified by northern blot as around 180 nt and by RACE as 179 nt which coincides with the rule that sRNAs range in size from 40 to 500 nt in length (Wang et al., 2009; Waqas et al., 2018). Conservation analysis indicated that *srvg17985* was a specific sRNA of *Vibrio* with simailar to *srvg23535*, and existed in *V. alginolyticus*, *V. natriegens*, *V. diabolicus*, *V. campbellii*, *V. harveyi*, *V. tasmaniensis* and *V. crassostreae* which is different from *srvg23535* as *srvg23535* is conserved among *V. harveyi, Vibrio parahaemolyticus*, and *Vibrio splendidus*, but does not present in *V. natriegens*, *V. diabolicus*, *V. campbellii*, *V. tasmaniensis* and *V. crassostreae* (Deng et al., 2018). In addition, *srvg17985* was synteny conserved among the less distant species of *V. alginolyticus*, *V. natriegens*, *V. diabolicus*, *V. campbellii*, and *V. harveyi*. The right flanking locus appeared to be rearranged in the more distant species of *V. tasmaniensis* and *V. crassostreae*. Sequence conservation analysis showed the transcript and terminator were highly homologous among the seven *Vibrio* spp., while the promoter sequence was different. Additionally, an FNR-box was found in *V. alginolyticus* and *V. diabolicus*, but an IHF-box only in *V. alginolyticus*. Thus, although *srvg17985* probably has conserved target-binding regions in different species, it might regulate different processes and may be expressed differently among these species (Holmqvist et al., 2012).

In *V. alginolyticus*, expression of *srvg17985* was induced during growth in both LBS and Zobell medium, similar to other sRNAs (Vogel

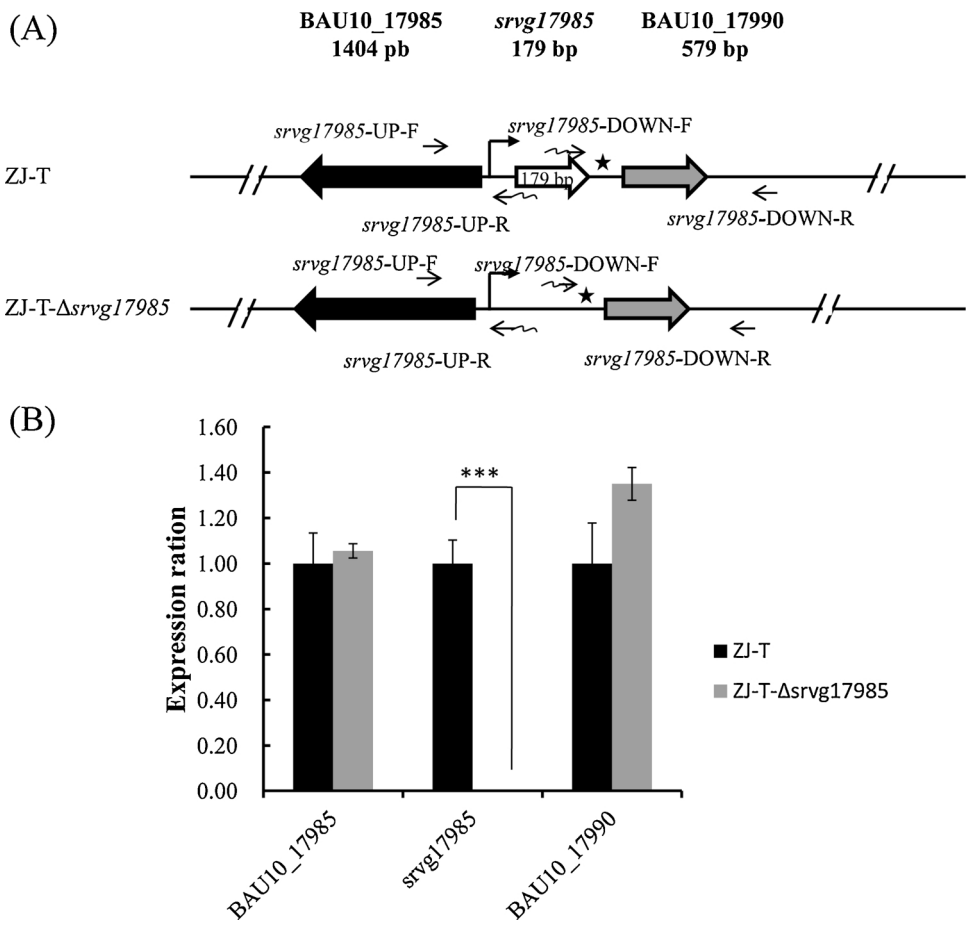

Fig. 4. Location and expression of *srvg17985* and its flanking genes in wild type and mutant strains. (A) *Srvg17985* was flanked by BAU10_17985 and BAU10_17990. The σ70 promoter is indicated with an arrow and the ρ-independent terminator with a star. The sequence from the 5' end to 38 bp before the 3' end was deleted in ZJ-T-Δ*srvg17985*. (B) RT-PCR analysis of *srvg17985*, BAU10_17985 and BAU10_17990, normalized to wild type levels. Values are mean ± SEM (n = 3). Statistically significant differences: ***$p < 0.001$.

Table 3
Gained and lost phenotypes of *srvg17985* mutant.

Mode of action	Test	Phenotype of *srvg17985* mutant
C-Source, amino acid	Gly-Glu	Lost
N-Source, amino acid	L-Phenylalanine	Gained
Osmotic sensitivity	9% NaCl	Gained
Osmotic sensitivity	10% NaCl	Gained
Osmotic sensitivity	3% Urea	Lost
Osmotic sensitivity	4% Urea	Lost
pH, deaminase	pH 9.5 + L-Serine	Gained
beta-D-glucuronidase	X-beta-D-Glucuronide	Lost

and Papenfort, 2006; Nguyen and Jacq, 2014). For example, several membrane stresses respond sRNAs, including *micA*, *omrA*, and *rybB* are induced in stationary phase (Papenfort et al., 2006; Vogel and Papenfort, 2006). CsrBs are produced at high cell density to control carbon metabolism, motility, quorum sensing, and biofilm formation, thus affecting pathogenicity (Nguyen and Jacq, 2014). In addition, the quorum sensing-regulating gene *cqsA* (BAU10_18875) and biofilm formation-regulating gene *gcvA* (BAU10_21995) were predicted as targets of *srvg17985*. These results indicated that *srvg17985* may be functional phase-dependent and participate in quorum sensing and biofilm formation in *V. alginolyticus*. Moreover, an FNR-box and an IHF box were found in the promoter of *srvg17985*. FNR is a fumarate and nitrate reductase regulatory protein that is anaerobically induced to bind to an FNR-box in the promoter to promote the transcription of genes involved in respiration, transmembrane, and anaerobic catabolism (Constantinidou et al., 2006). Fantappiè et al. (2011) found that the sRNA *aniS* of *Neisseria meningitides* is anaerobically induced through activation of its promoter by the FNR global regulator and is important in anaerobic adaptation. IHF typically changes along the growth curve and is critical for the transition from exponential to stationary phase (Azam et al., 1999; Valls et al., 2002). IHF binds the IHF-box in promoters and activates expression of corresponding genes to regulate generic architecture (recombination, translation, and transcription) and biological processes (motility) (Silva-Rocha et al., 2013; Lee and Zhao, 2015). Lee et al. found (2015) that the sRNA *rsmB* is positively regulated by IHF and controls motility. These results explained the induced expression mode of *srvg17985* during growth and indicated that *srvg17985* probably was anaerobically induced, since as bacteria grow, oxygen declines in the medium. *Srvg17985* was important for the transition from exponential to stationary phase.

PM assays indicated that *srvg17985*: (i) positively regulated the importation and/or degradation of Gly-Glu [degraded into glycyl (Gly) and L-glutamic acid (Glu)], but not the catabolism of Gly or Glu as *srvg17985* did not affect the metabolism of L-glutamic acid, Gly-Asp, Ala-Gly, or Gly-Pro; (ii) negatively regulated the importation, degradation and/or catabolism of L-phenylalanine; (iii) inhibited sodium ion efflux system while promoting urea transportation and/or hydrolysis; and (iv) negatively regulated the biosynthesis of L-serine deaminase (SdaA) at pH 9.5, affecting the neutralization of alkaline medium but positively regulating the expression of beta-D-glucuronidase (UidA). *Srvg17985* thus affected the hydrolysis of X-beta-D-glucuronide by adjusting the medium pH. HemB and MenD are involved in urea sensitivity and Gly-Glu metabolism as carbon sources in *Staphylococcus aureus*, shown with PM methods (von Eiff et al., 2006). The phenylalanine-4-hydroxylase-encoding gene *phhA*, regulating phenylalanine catabolism, and amidohydrolase-encoding gene *nit*, regulating urea hydrolysis, were detected in the ZJ-T genome (Deng et al., 2016b). In addition, two-component systems have been widely reported to regulate the response to osmotic stress and pH stress (Hyytiäinen et al., 2003; Tipton and Rather, 2017). The ABC transporter system is

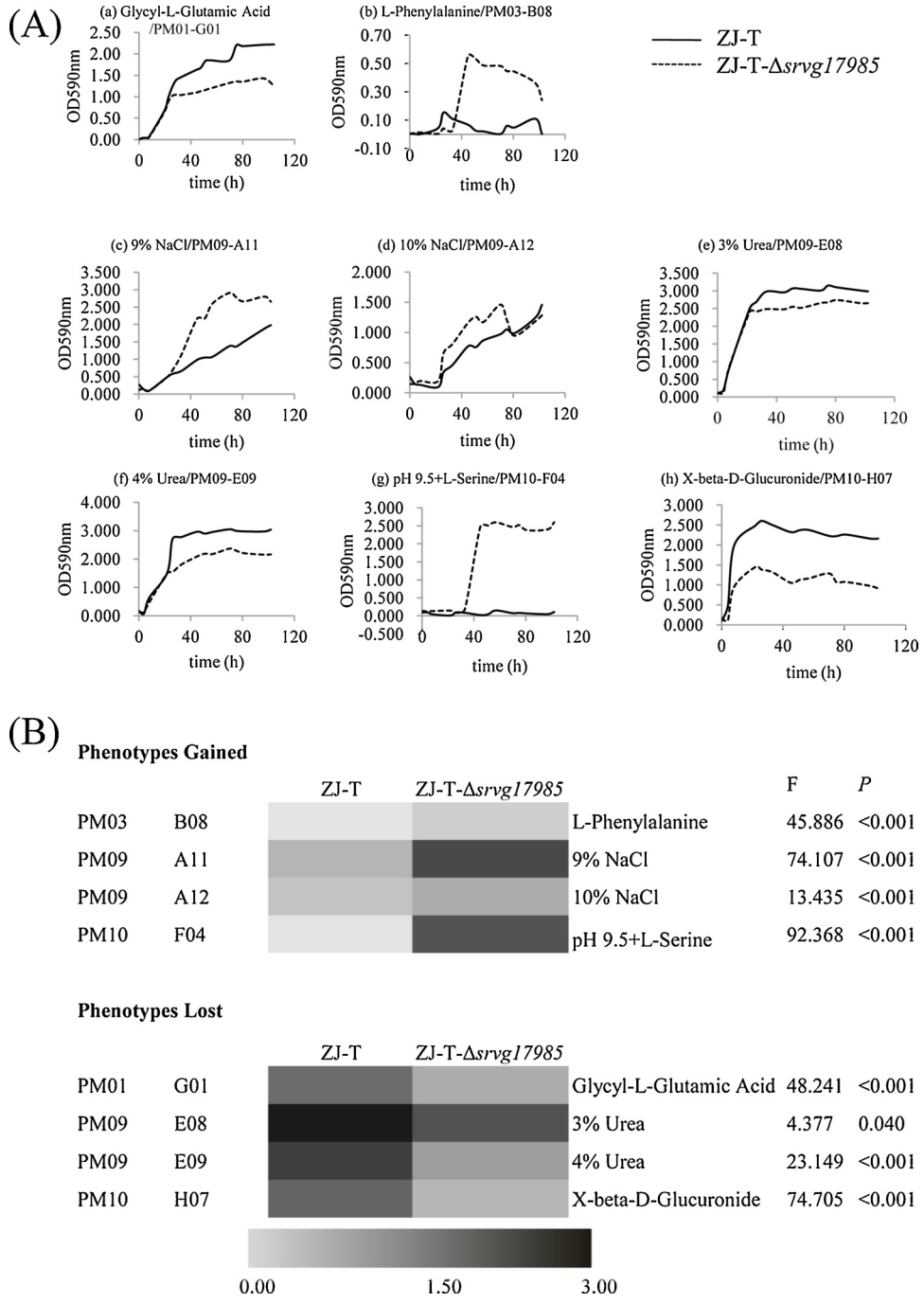

Fig. 5. Phenotypic characterization of *srvg17985* mutant. (A) Significantly different phenotypes. Shown are average values, n = 3. (B) Heat map of gained and lost phenotypes. F and *P* values are from one-way ANCOVA analysis.

reported to be involved in the osmotic stress response (van der Heide et al., 2001). For example, the two-component systems of RcsB (RR) and YojN (Hpt) are reported to regulate NaCl sensitivity in *E. coli* using PM methods (Zhou et al., 2003). Furthermore, Na^+/H^+ antiporters are critical for maintaining intracellular pH, cell volume, and osmotic homeostasis (Hunte et al., 2005). These results suggest that the genes of *hemB*, *menD*, *phhA*, *nit*, *sdaA*, and *uidA*, Na^+/H^+ antiporters, two-component systems, and the ABC transporter system could be regulated by *srvg17985*. *Srvg17985* might therefore affect the physiological processes of metabolism, osmotic sensitivity, and pH response. Five metabolism-related genes (BAU10_07995, BAU10_08780, BAU10_22375, BAU10_02050, and BAU10_06225), one ABC transporter permease-encoding gene (BAU10_17155) and one two-component system-related gene (BAU10_08620) were bioinformatically predicted as targets of *srvg17985* and probably contributed to the observed phenotypes. We note that urea hydrolysis is widely reported to be related to the potential pathogenicity of *Vibrio* spp. (Oberhofer and Podgore, 1982; Kaysner et al., 1994). The absence of *srvg17985* led to weaker tolerance to urea, indicating that *srvg17985* was involved in the pathogenicity of *V. alginolyticus*.

In conclusion, we comprehensively identified and characterized sRNA *srvg17985* for the first time in *V. alginolyticus*. It is specific to *Vibrio* spp. and consisted of five stem-loops. *Srvg17985* was induced by growth and very likely is critical for anaerobic and stationary adaptation. Moreover, *srvg17985* regulated the metabolism of some carbon and nitrogen sources and the response to several osmotic and pH

stresses, which are important for environmental adaptation and probably affect bacterial pathogenesis. This study supplies new evidence about the relationship between environmental adaptation and bacterial pathogenesis regulated by a sRNA of *V. alginolyticus*. Target prediction was conducted to provide new targets for antibacterial therapy or attenuated vaccines against *Vibrio* spp. For future studies, we suggest focusing on these two topics: (i) The interactions between *srvg17985* and the predicted target genes, for verification. Comparative RNA-seq is recommended to find more targets to fully understand the sRNA-mediated regulatory mechanism. (ii) As *srvg17985* is induced with growth and likely important in anaerobic adaptation and the transition from exponential to stationary phase, the regulation of FNR and IHF on *srvg17985* should be studied.

Author contributions

YD conceived the study, analyzed the data, and wrote the manuscript; YS and LB performed the experiments; SL and ZG critically revised the manuscript; HL, JF and CC contributed the reagents. All authors read and approved the manuscript for publication.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

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References

Azam, T.A., Iwata, A., Nishimura, A., Ueda, S., Ishihama, A., 1999. Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. J. Bacteriol. 181, 6361–6370.

Bak, G., Lee, J., Suk, S., Kim, D., Lee, J.Y., Kim, K.-s., Choi, B.S., Lee, Y., 2015. Identification of novel sRNAs involved in biofilm formation, motility, and fimbriae formation in *Escherichia coli*. Sci. Rep. 5, 15287.

Blumenstein, K., Macaya-Sanz, D., Martín, J.A., Albrectsen, B.R., Witzell, J., 2015. Phenotype MicroArrays as a complementary tool to next generation sequencing for characterization of tree endophytes. Front. Microbiol. 6, 1033.

Bochner, B.R., Gadzinski, P., Panomitros, E., 2001. Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. Genome Res. 11, 1246–1255.

Chen, C., Xie, J., Hu, C.Q., 2009. Phenotypic and genetic differences between opaque and translucent colonies of *Vibrio alginolyticus*. Biofouling 25, 525–531.

Constantinidou, C., Hobman, J.L., Griffiths, L., Patel, M.D., Penn, C.W., Cole, J.A., Overton, T.W., 2006. A reassessment of the FNR regulon and transcriptomic analysis of the effects of nitrate, nitrite, NarXL, and NarQP as *Escherichia coli* K12 adapts from aerobic to anaerobic growth. J. Biol. Chem. 281, 4802–4815.

Deng, Y., Chen, C., Zhao, Z., Zhao, J., Jacq, A., Huang, X., Yang, Y., 2016a. 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., Chen, C., Zhao, Z., Huang, X., Yang, Y., Ding, X., 2016b. Complete genome sequence of *Vibrio alginolyticus* ZJ-T. Genome Announc. 4, e00912–16.

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 *srvg23535* in *Vibrio alginolyticus* ZJ-T and its characterization with Phenotype MicroArray technology. Front. Microbiol. 9, 2394.

Fantappiè, L., Oriente, F., Muzzi, A., Serruto, D., Scarlato, V., Delany, I., 2011. A novel Hfq-dependent sRNA that is under FNR control and is synthesized in oxygen limitation in *Neisseria meningitidis*. Mol. Microbiol. 80, 507–523.

Fröhlich, K.S., Gottesman, S., 2018. Small regulatory RNAs in the *Enterobacterial* response to envelope samage and oxidative stress. Microbiol. Spectr. 6.

Gardner, P.P., Daub, J., Tate, J.G., Nawrocki, E.P., Kolbe, D.L., Lindgreen, S., Wilkinson, A.C., Finn, R.D., Griffiths-Jones, S., Eddy, S.R., Bateman, A., 2008. Rfam: updates to the RNA families database. Nucleic Acids Res. 37, D136–D140.

He, H., Wang, Q., Sheng, L., Liu, Q., Zhang, Y., 2011. Functional characterization of *Vibrio alginolyticus* twin-arginine translocation system: its roles in biofilm formation, extracellular protease activity, and virulence towards fish. Curr. Microbiol. 62, 1193–1199.

Holmqvist, E., Unoson, C., Reimegård, J., Wagner, E.G.H., 2012. A mixed double negative feedback loop between the srna *micf* and the global regulator *lrp*. Mol. Microbiol. 84 (3), 414–427.

Huang, L., Hu, J., Su, Y., Qin, Y., Kong, W., Ma, Y., Xu, X., Lin, M., Yan, Q., 2015. Identification and characterization of three *Vibrio alginolyticus* non-coding RNAs involved in adhesion, chemotaxis, and motility processes. Front. Cell. Infect. Microbiol. 5, 56.

Huang, X., Chen, C., Ren, C., Li, Y., Deng, Y., Yang, Y., Ding, X., 2017. Identification and characterization of a locus putatively involved in colanic acid biosynthesis in *Vibrio alginolyticus* ZJ-51. Biofouling 34, 1–14.

Hunte, C., Screpanti, E., Venturi, M., Rimon, A., Padan, E., Michel, H., 2005. Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH. Nature 435, 1197.

Hyytiäinen, H., Sjöblom, S., Palomäki, T., Tuikkala, A., Tapio Palva, E., 2003. The PmrA-PmrB two-component system responding to acidic pH and iron controls virulence in the plant pathogen *Erwinia carotovora* ssp. carotovora. Mol. Microbiol. 50, 795–807.

Kang, Z., Zhang, C., Zhang, J., Jin, P., Zhang, J., Du, G., Chen, J., 2014. Small RNA regulators in bacteria: powerful tools for metabolic engineering and synthetic biology. Appl. Microbiol. Biotechnol. 98, 3413–3424.

Kaysner, C.A., Abeyta, C., Trost, P.A., Wetherington, J.H., Jinneman, K.C., Hill, W.E., Wekell, M.M., 1994. Urea hydrolysis can predict the potential pathogenicity of *Vibrio parahaemolyticus* strains isolated in the Pacific Northwest. Appl. Environ. Microbiol. 60, 3020–3022.

Kery, M.B., Feldman, M., Livny, J., Tjaden, B., 2014. TargetRNA2: identifying targets of small regulatory RNAs in bacteria. Nucleic Acids Res. 42, W124–W129.

Klein, G., Raina, S., 2017. Small regulatory bacterial RNAs regulating the envelope stress response. Biochem. Soc. Trans. 45, 417–425.

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, 777–784.

Lee, J.H., Zhao, Y., 2015. Integration host factor is required for RpoN-dependent *hrpL* gene expression and controls motility by positively regulating *rsmB* sRNA in *Erwinia amylovora*. Phytopathology 106, 29–36.

Liu, W., Rochat, T., Toffano-Nioche, C., Lam, L., Nguyen, T., Bouloc, P., Morvan, C., 2018. Assessment of bona fide sRNAs in *Staphylococcus aureus*. Front. Microbiol. 9, 228.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Loris, R., Dao-Thi, M.H., Bahassi, E.M., Melderen, L.V., Poortmans, F., Liddington, R., Couturier, M., Wyns, L., 1999. Crystal structure of CcdB, a topoisomerase poison from *E. coli* 1. J. Mol. Biol. 285 (4), 1667–1677.

Lu, P., Wang, Y., Hu, Y., Chen, S., 2018. RgsA, an RpoS-dependent sRNA, negatively regulates *rpoS* expression in *Pseudomonas aeruginosa*. Microbiology 164 (4), 716–724.

Melamed, S., Peer, A., Faigenbaum-Romm, R., Gatt, Y.E., 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.

Naville, M., Ghuillot-Gaudeffroy, A., Marchais, A., Gautheret, D., 2011. ARNold: a web tool for the prediction of Rho-independent transcription terminators. RNA Biol. 8, 11–13.

Nguyen, A.N., Disconzi, E., Charrière, G.M., Destoumieux-Garzón, 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, e00582–00518.

Nguyen, A.N., Jacq, A., 2014. Small RNAs in the *Vibrionaceae*: an ocean still to be explored. Wiley Interdiscip. Rev. RNA 5, 381–392.

Oberhofer, T.R., Podgore, J.K., 1982. Urea-hydrolyzing *Vibrio parahaemolyticus* associated with acute gastroenteritis. J. Clin. Microbiol. 16, 581–583.

Pérez-Reytor, D., Plaza, N., Espejo, R.T., Navarrete, P., Bastías, R., Garcia, K., 2017. Role of non-coding regulatory RNA in the virulence of human pathogenic vibrios. Front. Microbiol. 7, 2160.

Papenfort, K., Pfeiffer, V., Mika, F., Lucchini, S., Hinton, J.C., Vogel, J., 2006. σ^E-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global *omp* mRNA decay. Mol. Microbiol. 62, 1674–1688.

Papenfort, K., Vogel, J., 2014. Small RNA functions in carbon metabolism and virulence of enteric pathogens. Front. Cell. Infect. Microbiol. 4, 91.

Schindelin, J., Rueden, C.T., Hiner, M.C., Eliceiri, K.W., 2015. The ImageJ ecosystem: an open platform for biomedical image analysis. Mol. Reprod. Dev. 82, 518–529.

Silva-Rocha, R., Chavarría, M., Kleijn, R.J., Sauer, U., de Lorenzo, V., 2013. The IHF regulon of exponentially growing *Pseudomonas putida* cells. Environ. Microbiol. 15,

49–63.
Silveira, A.C.G., Robertson, K.L., Lin, B., Wang, Z., Vora, G.J., Vasconcelos, A.T.R., 2010. Identification of non-coding RNAs in environmental vibrios. Microbiology 156, 2452–2458.
Siqueira, F.M., Morais, G.L.D., Higashi, S., Beier, L.S., Breyer de Sá Godinho, C.P., Sagot, M.F., Schrank, I.S., Zaha, A., de Vasconcelos, A.T., 2016. Mycoplasma non-coding RNA: identification of small RNAs and targets. BMC Genomics 17 (8), 743.
Solovyev, V.V., Shahmuradov, I.A., Salamov, A.A., 2010. Identification of promoter regions and regulatory sites. Computational Biology of Transcription Factor Binding. pp. 57–83.
Storz, G., Vogel, J., Wassarman, K.M., 2011. Regulation by small RNAs in bacteria: expanding frontiers. Mol. Cell 43, 880–891.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Thompson, J.D., Gibson, T.J., Higgins, D.G., 2003. Multiple sequence alignment using ClustalW and ClustalX. Curr. Protoc. Bioinf. 2–3.
Tipton, K.A., Rather, P.N., 2017. An *ompR-envZ* two-component system ortholog regulates phase variation, osmotic tolerance, motility, and virulence in *Acinetobacter baumannii* strain AB5075. J. Bacteriol. 199, e00705–16.
Valls, M., Buckle, M., de Lorenzo, V., 2002. In vivo UV laser footprinting of the *Pseudomonas putida* ς^{54} Pu promoter reveals that integration host factor couples transcriptional activity to growth phase. J. Biol. Chem. 277, 2169–2175.
Val, M.-E., Skovgaard, O., Ducos-Galand, M., Bland, M.J., Mazel, D., 2012. Genome engineering in *Vibrio cholerae*: a feasible approach to address biological issues. PLoS Genet. 8, e1002472.
van der Heide, T., Stuart, M.C., Poolman, B., 2001. On the osmotic signal and osmosensing mechanism of an ABC transport system for glycine betaine. EMBO J. 20, 7022–7032.
Vogel, J., Papenfort, K., 2006. Small non-coding RNAs and the bacterial outer membrane. Curr. Opin. Microbiol. 9, 605–611.
von Eiff, C., McNamara, P., Becker, K., Bates, D., Lei, X.H., Ziman, M., Bochner, B.R., Peters, G., Proctor, R.A., 2006. Phenotype microarray profiling of *Staphylococcus aureus menD* and *hemB* mutants with the small-colony-variant phenotype. J. Bacteriol. 188, 687–693.
Wagner, E.G.H., Romby, P., 2015. Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. Adv. Genet. 90, 133–208.
Wang, L., Zhao, Y., Li, W., 2009. Research progress of prediction of bacterial sRNA genes and their targets—a review. Wei Sheng Wu Xue Bao 49 (1), 1–5.
Waqas, A., Mian, A.H., Sammina, M., 2018. Identification and functional characterization of bacterial small non-coding RNAs and their target: a review. Gen. Rep. 10, 167–176.
Ye, J., Ma, Y., Liu, Q., Zhao, D., Wang, Q., Zhang, Y., 2008. Regulation of *Vibrio alginolyticus* virulence by the LuxS quorum-sensing system. J. Fish Dis. 31, 161–169.
Zhou, L., Lei, X.H., Bochner, B.R., Wanner, B.L., 2003. Phenotype microarray analysis of *Escherichia coli* k-12 mutants with deletions of all two-component systems. J. Bacteriol. 185, 4956–4972.
Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.