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DUF1127-containing protein and ProQ had opposite effects on biofilm formation in *Vibrio alginolyticus*

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Abstract

The RNA binding protein is crucial for gene regulation at the post transcription level. In this study, functions of the DUF1127-containing protein and ProQ, which are RNA-binding proteins, were revealed in *Vibrio alginolyticus*. DUF1127 deletion increased the ability of biofilm formation, whereas ProQ deletion reduced the amount of biofilm. Moreover, extracellular proteinase secretion was significantly reduced in the DUF1127 deletion strain. ProQ, not DUF1127-containing protein, can help the cell to defense oxidative stress. Deletion of DUF1127 resulted in a higher ROS level in the cell, however, ProQ deletion showed no difference. RNA-seq unveiled the expression of genes involved in extracellular protease secretion were significantly downregulated and biofilm synthesis-related genes, such as *rbsB* and *alsS*, were differentially expressed in the DUF1127 deletion strain. ProQ affected the expression of genes involved in biofilm synthesis (*flgC* and *flgE*), virulence (*betB* and *hutG*), and oxidative stress. Moreover, the DUF1127-containing and ProQ affected the mRNA levels of various regulators, such as LysR and BetI. Overall, our study revealed that the DUF1127-containing protein and ProQ have crucial functions on biofilm formation in *V. alginolyticus*.

Keywords *Vibrio alginolyticus*, RNA-binding protein, ProQ, DUF1127, Biofilm

Introduction

V. alginolyticus is a gram-negative bacterium widely distributed in aquatic environments, including oceans and estuaries [1, 2]. Being a conditional pathogenic bacterium, *V. alginolyticus* possesses the regulatory ability to resist stresses from the environment and the host. Gene expression regulation at transcriptional and post-transcriptional levels help bacterial adaptation to various environments [3, 4]. Post-transcriptional regulation involves gene expression regulation at the RNA level, which occurs in a cell throughout its entire lifespan [4]. Various post-transcriptional regulatory factors have currently been identified in bacteria, including RNA-binding proteins (RBPs) and small RNAs (sRNAs) [5, 6]. sRNAs are usually noncoding RNAs (ncRNAs) that can bind to their target mRNAs, ultimately inhibiting or activating translation [7].

RBPs can bind to RNAs including sRNAs in cells [8, 9], which play regulatory roles in post-transcriptional processes such as splicing, modification, cellular localization, stability, and RNA degradation [6, 10, 11]. Hfq is a well-studied RBP that exists in various bacteria and plays

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a crucial role in gene expression regulation by facilitating sRNA–target mRNA pairing in cells [12–14]. ProQ is another critical RBP with a Fino domain and various functions in bacteria. ProQ can directly bind to sRNA regions by overlapping with RNase targets through complementary base pairing, which results in a more stable sRNA [15]. For example, in *Salmonella enterica*, ProQ protects *cspE* mRNA from RNase II-mediated degradation [16]. Additionally, ProQ preferentially binds to RNA stem-loop structures and interacts with rho-independent terminators [17]. It also exhibits higher affinity for terminators with A-rich sequences on the 5′ side [18]. Moreover, it can bind to sRNAs and thus regulates the expression of related target genes [16, 19]. Many novel RBPs have recently been identified, such as RbpA; RbpA has a role in the pathogenicity of *Streptococcus suis* serotype 2 [20]. A DUF1127-containing protein named as CcaF1 was proved to be an RBP in *Rhodobacter sphaeroides*, CcaF1 primarily controls RNA levels by affecting the stability of mature transcripts involved in sRNA maturation and RNA turnover [21]. In *Salmonella*, the DUF1127-containing protein named as YjiS is involved in bacterial phosphate and carbon metabolism and is also associated with bacterial virulence [22].

Biofilm is among the key virulence factors of *V. alginolyticus* that assists the bacteria in resisting various environmental stresses and provides a pathway for acquiring nutrition [23, 24]. For bacteria, biofilm formation is a complex process. Based on external environmental conditions, the intracellular regulatory network needs to coordinate biofilm formation and dispersion. This regulatory network involves various factors, including quorum sensing (QS), second messengers, sRNAs, and transcriptional factors [25]. In *V. alginolyticus*, *rpoN* deletion caused flagellar defects, and therefore, the bacteria could not detach from the biofilm and become single cells [26]. In *Bacillus subtilis*, *hfq* gene knockout inhibited the expression of most QS-related genes and led to reduced biofilm formation [27]. In *S. enterica*, sRNA1186573 is essential for biofilm formation [28].

In our previous study, the expressions of genes encoding a DUF1127-containing protein (WP_224721253.1) and ProQ (WP_005377435.1) in *V. alginolyticus* were induced by oxidative stress [29]. ProQ is a well-studied RBP; however, its function in *V. alginolyticus* remains unknown. DUF1127-containing protein is a newly identified RBP in bacteria, and information about its function remains limited. We here reveal the functions of the DUF1127-containing and ProQ in *V. alginolyticus*. The study results offer novel insights into the post-transcriptional regulatory roles of the DUF1127-containing and ProQ in bacteria.

Materials and methods

Strains and bacterial culture

Table S1 presents a detailed list of all plasmids and bacterial strains used in this study. Table S2 lists the primer sequences used. *V. alginolyticus* and *Escherichia coli* were grown at 25 °C and 37 °C in Luria-Bertani (LB) broth or LB medium containing 1.5% (w/v) agar, respectively. Ampicillin or chloramphenicol was added to the liquid and solid media when necessary.

Deletion of the DUF1127-containing protein and ProQ and overexpression

The nucleotide sequence encoding the DUF1127-containing protein was partially deleted using Liu et al.'s method [30]. In short, the upstream and downstream fragments of the knockout region were amplified using the primers DUF100-U-F and DUF100-U-R, and DUF100-D-F and DUF100-D-R, respectively. A 50-bp overlap between the two fragments was fused using the primers DUF100-U-F and DUF100-D-R. This resulted in a fragment that was ligated into the suicide vector pDM4. The resulting plasmid, pDM4_D100UD, was transformed into *E. coli* S17-1 and transferred into the wild-type (WT) strain through conjugation. The transconjugants were selected on LB agar plates containing ampicillin (100 μg/mL) and chloramphenicol (100 μg/mL). DUF1127 deletion mutants were screened on LB plates containing 10% sucrose and verified through PCR by using the primers DUF100-U-F and DUF100-D-R. The nucleotide sequence encoding the DUF1127-containing protein (201 bp) was amplified using the primers DUF-O-F and DUF-O-R and ligated into the plasmid pMMB207. The resulting plasmid, pMMB207_DUF1127, was transferred into the WT strain through conjugation. The transconjugants were selected on LB agar plates containing ampicillin and chloramphenicol. DUF1127 overexpression strain was confirmed through RT-PCR by using appropriate primers.

proq deletion and overexpression strains were constructed using the same method. The upstream and downstream fragments were amplified using the primers ProQ-U-F and ProQ-U-R, and ProQ-D-F and ProQ-D-R, respectively. Then, both the fragments were fused using the primers ProQ-U-F and ProQ-D-R and ligated into the plasmid pDM4. The remaining steps followed were the same as described above to obtain the ProQ mutant strain. Similarly, the *proq* fragment was amplified using the primers ProQ-O-F and ProQ-O-R, ligated into the plasmid pMMB207, and transformed into the WT strain by using the same aforementioned steps to obtain the ProQ overexpression strain.

Measurement of extracellular proteinase

The partially modified method of a previous study was used for measuring extracellular proteinase [31]. The 2× LB solid medium (with all components doubled compared with the LB medium) and 30% skim milk powder were prepared, mixed in equal volumes, poured into culture dishes, and prepared as skim milk plates. The strains were cultured to the exponential phase (OD_{600nm}=0.6) and spotted onto the skim milk plate, which was incubated upright at 25 °C. The clear zone was observed at 24 h and 48 h. Five independent experiments were conducted for each condition.

Measurement of ROS level

100 mL of semi-solid LB medium containing 0.3% agar was prepared in advance and kept in a liquid state at 30℃. The strains were cultured to the exponential period (OD_{600nm}=0.6). Then, 10 mL culture was taken and thoroughly mixed with the aforementioned medium. Subsequently, 5 ml of the mixed bacterial suspension was applied onto solid LB medium. After solidification, a perforated paper disc was placed in the center and 1 µl of 20% H_2O_2 was added. The culture was incubated upright at 25 °C for 24 h, and the diameter of the transparent zone was measured using a caliper. Five independent experiments were performed for each condition.

Detection of biofilm formation

The partially modified method of a previous study was used for detecting biofilm formation [26]. After the strains were cultured to the exponential phase (OD_{600nm}=0.6) at 25℃, 1 mL bacterial inoculum was diluted 5-fold with LB medium and added to a culture dish. The mixture was statically incubated at 25℃ for 48 h. This experiment was repeated 3 times independently. The bacterial cells were washed with flowing distilled water, fixed with methanol, and stained with 1% crystal violet for 15 min before the photos were taken. The crystal violet-stained cells were fully dissolved in 95% ethanol. Finally, the absorbance was measured at 570 nm.

Moreover, the biofilm was also observed under a scanning electron microscope [32]. Upon reaching the exponential period (OD_{600nm}=0.6), the bacterial inoculum was diluted 5-fold with LB and added to a culture dish containing a sterile glass slide. Then, the mixture was statically incubated at 25 °C for 48 h. After cultivation was complete, the planktonic bacteria were gently aspirated, and the biofilm was fixed with 2.5% glutaraldehyde. The samples were then dehydrated sequentially using 30%, 50%, 70%, 80%, and 90% absolute ethanol, frozen, freeze-dried to obtain a dry state, gold sputtered at the Guangdong Technion-Israel Institute of Technology, and observed through scanning electron microscopy (SEM) (EHT: Extra high tension; WD: Working Distance; Mag: Magnification).

Measurement of cyclic dimeric guanosine monophosphate

After the bacteria were cultivated to the exponential phase (OD_{600nm}=0.6), the cells were harvested through centrifugation at 12,000 *g* for 1 min at 4 °C, washed with precooled PBS buffer, disrupted using an ultrasonic disruptor (break for 5 s, pause for 3 s), and centrifuged at 12,000 *g* for 10 min at 4 °C, to obtain the supernatant. The total protein concentration of half of the obtained protein samples was determined using the BCA assay kit (Beyotime Bioechnology, China). The cyclic dimeric guanosine monophosphate (c-di-GMP) level of the remaining half of the samples was determined using the Cyclic di-GMP ELISA Kit (Jiangsu Jingmei Biotechnology Co., Ltd). After the color reaction was complete, the absorbance was measured at 450 nm. The c-di-GMP level in the sample was calculated based on the standard curve.

RNA-seq analysis

After the mutant strain and WT strain were cultured to the exponential phase (OD_{600nm}=0.6), RNA was extracted using the Trizol method [33]. The extracted RNA was treated with DNase I to remove any genomic DNA contamination and subjected to PE150 sequencing by using the Illumina high-throughput sequencing platform. The obtained sequencing data were quality-controlled using fastp and compared with the ribosomal RNA sequences in the Rfam database by using Bowtie2 software to obtain data without rRNA [34]. BAM files and GTF (or GFF) annotation information were used, and read counts were used for differential gene analysis of multiple sample groups. The sequencing data were uploaded to SRA (NCBI) (accession numbers: PRJNA962541 and PRJNA1066051). sRNA was predicted using Rockhopper software, and sRNA target genes were predicted using IntaRNA software [35–38].

RT-qPCR

RT-qPCR was performed as described elsewhere [39]. In short, RNA was diluted to 500 ng/µL. RT-qPCR was conducted in a Roche fluorescence qPCR apparatus by using PerfectStart® Green qPCR SuperMix Kit (Beijing TransGen Biotech, China). The 16S rRNA was used as an internal control, and the data were processed using the $2^{-\Delta\Delta Ct}$ method [40].

Results

Functions of the DUF1127-containing protein and ProQ in oxidative stress resistance and extracellular proteinase secretion

In our previous study, oxidative stress was found to induce the expression of genes encoding a DUF1127-containing

protein and ProQ [41]. The function of the DUF1127-containing protein in *V. alginolyticus* remains unknown. Bioinformatic analysis revealed that the genome had only one gene encoding the DUF1127-containing protein (66 amino acids). The DUF1127-containing protein-encoding gene is connected to four sRNAs in *R. sphaeroides* [21]. However, our analysis unveiled that no sRNAs were predicted to be located at the downstream of the DUF1127 gene in *V. alginolyticus* (Fig. 1A). The DUF1127-containing protein from *R. sphaeroides* was similar to the Smaug protein from *Drosophila melanogaster*. The Smaug protein is involved in RNA turnover and in the development of fruit flies and mammals [39, 40]. The structure of the DUF1127-containing protein in *V. alginolyticus* was also predicted to be similar to that of the Smaug protein (Figure S1) (https://swissmodel.expasy.org/interactive). The results showed that there were conserved amino acids (isoleucine, leucine, and glycine) and SAM domains in DUF1127 proteins of *V. alginolyticus* and *R. sphaeroides* (Fig. 1B). Additionally, the FinO domain-containing ProQ is highly conserved in bacteria (Fig. 2A). Furthermore, in both bacterial strains, the *proq* gene is followed by *prc* (a carboxy terminal-processing peptidase) (Fig. 2B).

To identify and demonstrate the functions of the DUF1127-containing protein and ProQ in *V. alginolyticus*, we constructed DUF1127 and ProQ gene deletion strains and validated them through PCR (Figure S2). In the previous study, oxidative stress was found to induce the expression of the DUF1127-containing protein and

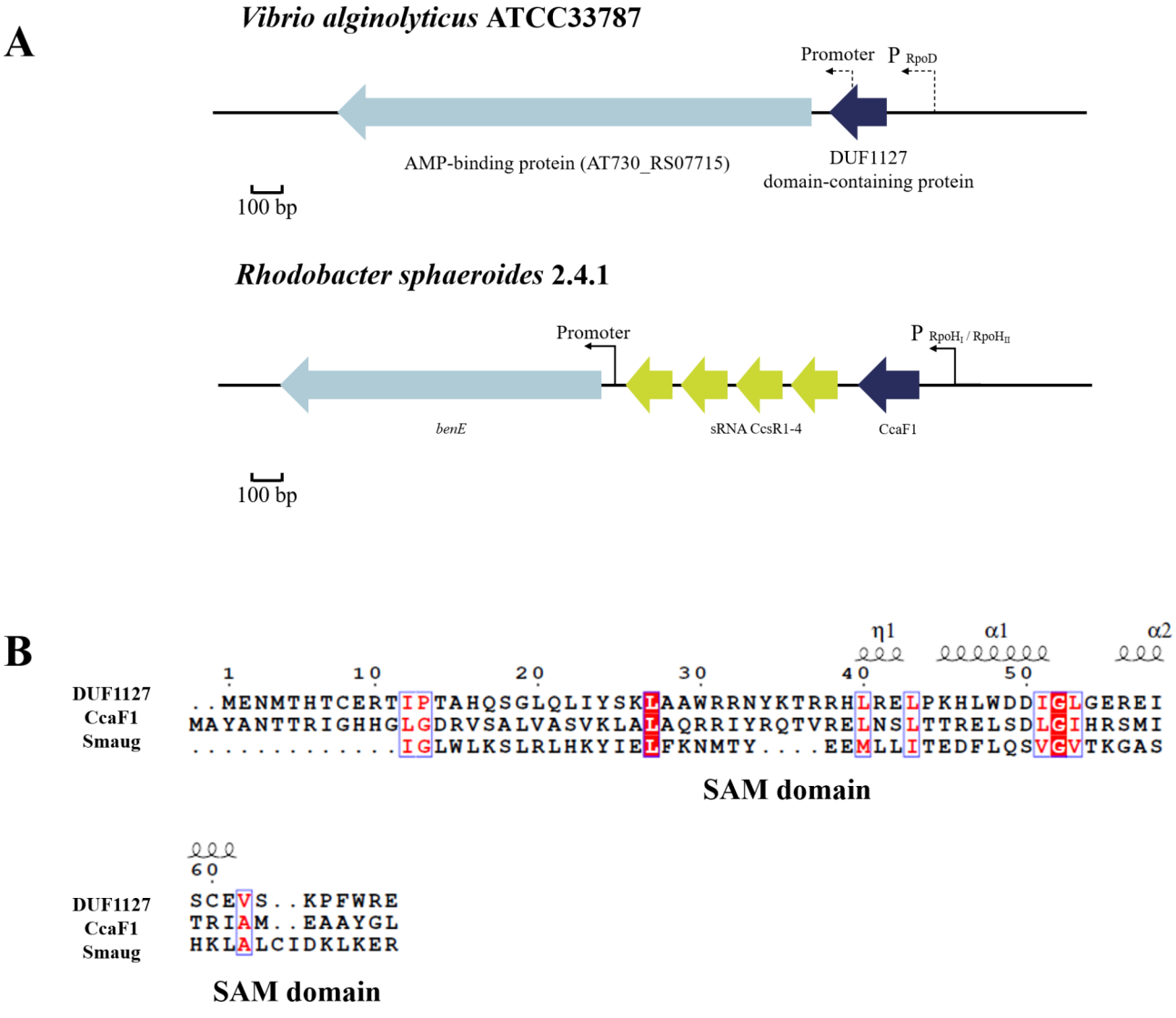

Fig. 1 Bioinformatics analysis of the DUF1127-containing protein. (**A**) The location of the DUF1127 gene in *Vibrio alginolyticus and Rhodobacter sphaeroides*. (**B**) Alignment of the DUF1127-containing protein sequence. Red highlights indicate the amino acids of ProQ conserved in *V. alginolyticus* and *E. coli*. The domain is positioned at the appropriate position

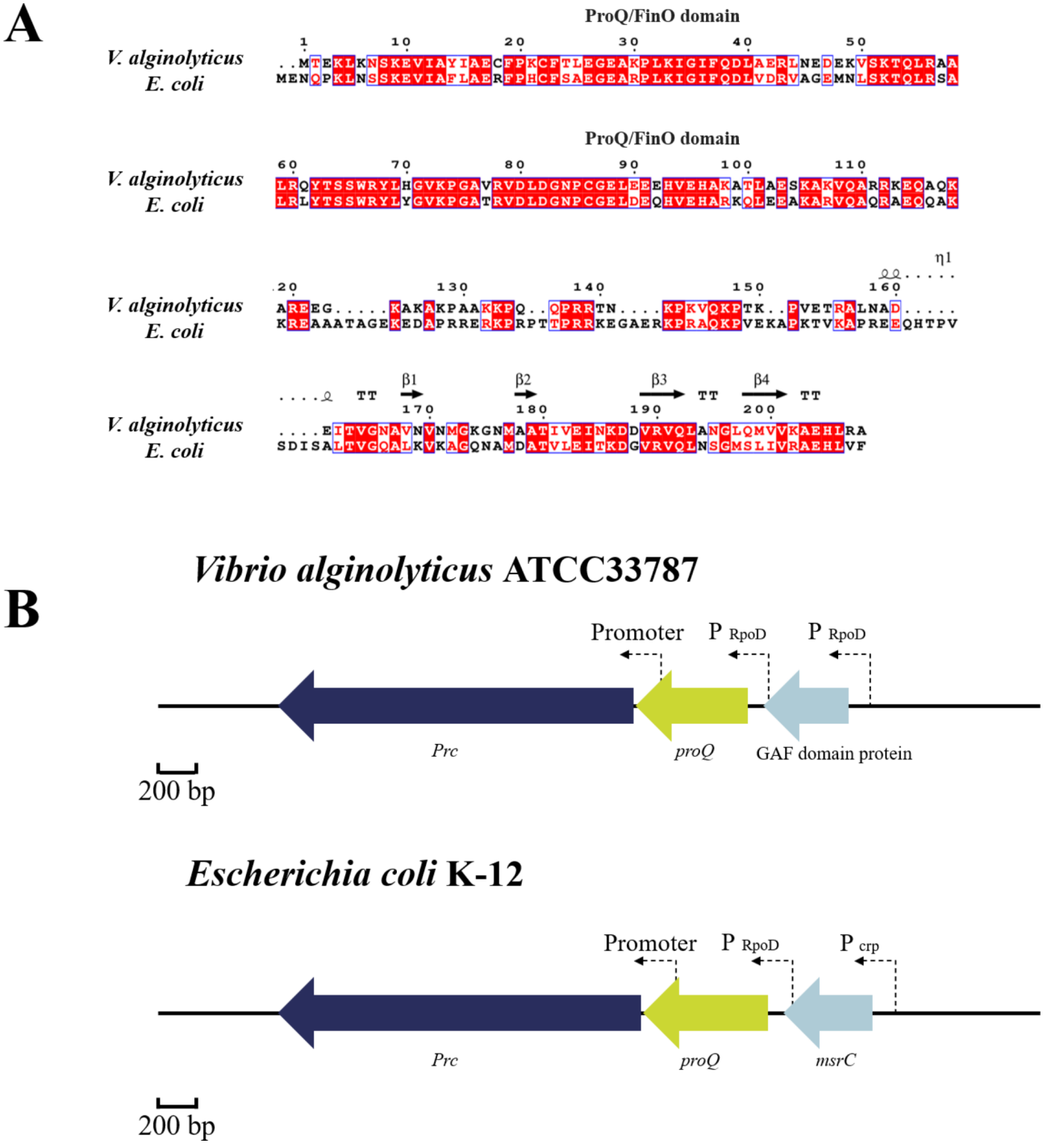

Fig. 2 Bioinformatics analysis of the ProQ protein. (**A**) Alignment of the ProQ protein sequence. The conserved amino acids are shown in red color. The domain is positioned at the appropriate position. (**B**) The location of the *proq* gene in *V. alginolyticus and R. sphaeroides*

ProQ. In this study, the ΔDUF1127 strain exhibited no difference in oxidative stress resistance compared with the WT strain (Figure S3). However, using the ROS Assay Kit (Beyotime Bioechnology, China), we observed that the reactive oxygen species (ROS) level decreased after DUF1127 was deleted in *V. alginolyticus* (Fig. 3A). The ROS level in the ΔProQ strain exhibited no difference compared with that in the WT strain (Figure S4). However, under H_2O_2 stress, the ΔProQ strain demonstrated less resistance to oxidative stress (Fig. 3B, Figure S5). In some studies, ProQ was found to be involved in the oxidative stress-resisting process in many bacteria, including *E. coli* and *Salmonella* [19, 42].

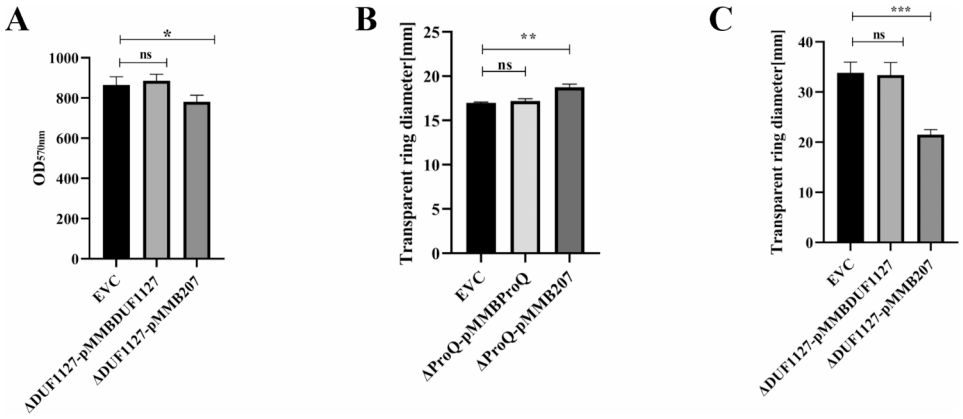

Fig. 3 Phenotype analysis. (**A**) DUF1127 increases the ROS level in *V. alginolyticus*. For each strain, three parallel samples were processed using a reagent kit. The absorbance at 570 nm was measured, and the absorbance values were proportional to intracellular ROS levels. (**B**) ProQ can help *V. alginolyticus* resist oxidative stress. The diameter of the inhibition zones on the plates was measured using a vernier caliper. Each plate was measured 3 times, and the data were recorded and plotted. (**C**) DUF1127 positively regulates extracellular protease secretion in *V. alginolyticus*. The diameter of the transparent rings on the plates was measured using a vernier caliper. Each plate was measured three times, and the data were recorded and plotted. Five biological replicates were included in each experiment. Means with asterisks are significantly different from those of the control, according to Student's t-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and ns means no significant difference

V. alginolyticus produces various virulence factors such as extracellular proteases. These factors can directly damage the host's immune defense system. In the present study, the effect of the DUF1127-containing protein on the secreted extracellular proteinase was investigated using the skim milk agar plate method. The level of the secreted extracellular proteinase was significantly reduced after DUF1127 deletion (Fig. 3C), indicating that the DUF1127-containing protein was involved in regulating the secretion of extracellular proteinase in *V. alginolyticus*.

The DUF1127-containing protein and ProQ exert opposite effects on biofilm formation in *V. alginolyticus*

In bacteria, biofilms help in resisting unfavorable external conditions and maintaining their growth and reproduction. To investigate whether the DUF1127-containing protein and ProQ were involved in biofilm formation, both the deletion strains were cultured for 48 h, and biofilm formation was analyzed in these strains. The amount of biofilm formed by the DUF1127 deletion strain was considerably higher than that of the WT strain (Fig. 4A). Additionally, SEM was performed to observe the biofilm at the cellular level after 24 h of culturing. The ΔDUF1127 strain had a higher stacking density than the WT strain, indicating that the DUF1127-containing protein exerted a negative regulatory effect on biofilm formation (Fig. 4C). Biofilm formation of the DUF1127 deletion strain was also increased in *Agrobacterium tumefaciens* [43].

Interestingly, the ProQ protein exerted a positive regulatory effect on biofilm formation (Fig. 4B). Crystal violet staining unveiled that the ΔProQ strain had a significantly lower capacity to form biofilm than the WT strain. Further validation through SEM supported this observation. In a panoramic view, large gaps were noted between bacterial cells of the biofilm in the ΔProQ strain. By contrast, the WT strain exhibited close inter-bacterial cell connections. A closer examination at a higher magnification unveiled that the ΔProQ strain, compared with the WT strain, barely formed any biofilm. No stacking was formed between the cells. This indicates that ProQ can promote biofilm formation in *V. alginolyticus* (Fig. 4C). A study reported ProQ to be essential for biofilm formation [44]. This result aligns with the function of ProQ in *V. alginolyticus*.

Genes and sRNAs affected by the DUF1127-containing protein and ProQ

To analyze genes affected by the DUF1127-containing protein, RNA-seq was performed using DUF1127 deletion strain compared with the WT strain. In total, 151 differentially expressed genes were identified. Overall, 79 genes ($\log_2$ fold change ≥ 0.5 and $P \leq 0.05$) were upregulated and 72 genes ($\log_2$ fold change ≤ -0.5 and $P \leq 0.05$) were significantly downregulated in the ΔDUF1127 strain (Fig. 5A). Table S3 summarizes details of all differentially expressed genes. Furthermore, to assess the accuracy of the RNA-seq data, several genes from the transcriptome were selected for validation using RT-qPCR. The results demonstrated a consistent trend between RT-qPCR and RNA-seq results (Figure S6). In the transcriptome of the ΔDUF1127 mutant strain, genes involved in extracellular protease secretion, such as serine protease (AT730_RS13155) and *cobA*, were significantly downregulated. Moreover, biofilm synthesis-related genes, such as *rbsB* and *alsS* [45, 46], and genes associated with the two-component system (AT730_RS18845), were significantly differentially expressed in the transcriptome.

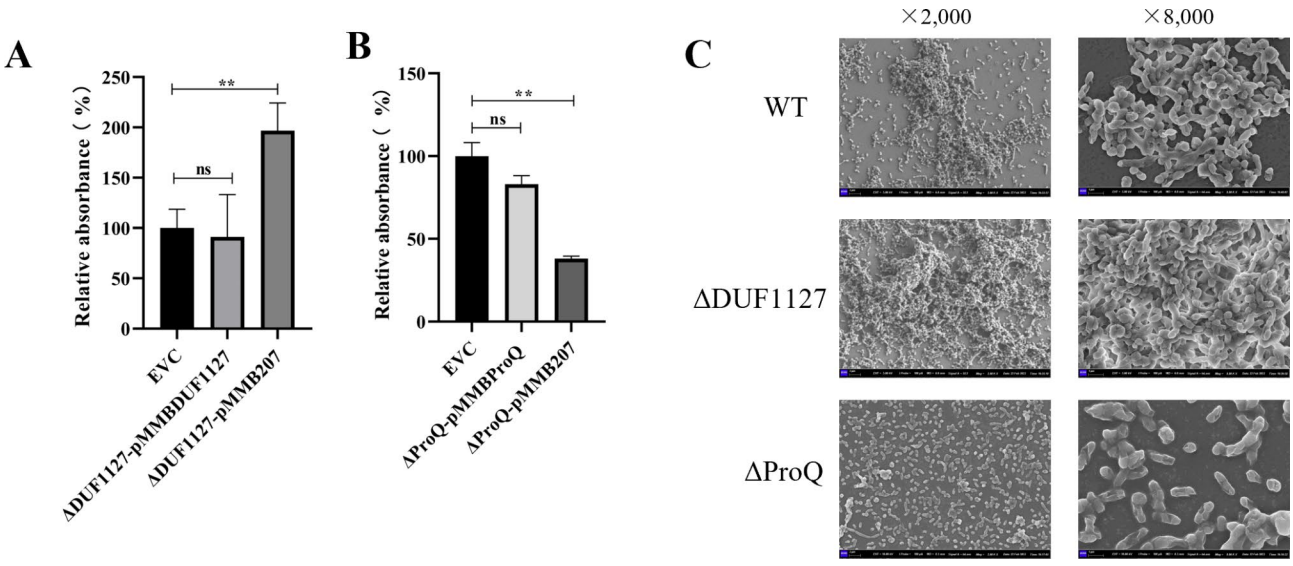

Fig. 4 DUF1127 and ProQ regulate biofilm formation. The biofilm was stained with crystal violet. After staining was completed, the biofilm was dissolved in ethanol. The absorbance measured at 570 nm was proportional to the biofilm content. (**A**) Assays of biofilm formation of EVC and ΔDUF1127-pMMB207. The relative absorbance value represents the percentage relative to the EVC absorbance value. (**B**) Assays of biofilm formation of EVC and ΔProQ-pMMB207. The relative absorbance value represents the percentage relative to the EVC absorbance value. (**C**) Scanning electron micrographs. WT and ΔDUF1127 (×2,000, ×8,000 magnifcation). Means with asterisks are significantly different from those of the control, according to Student's t-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and ns means no significant difference

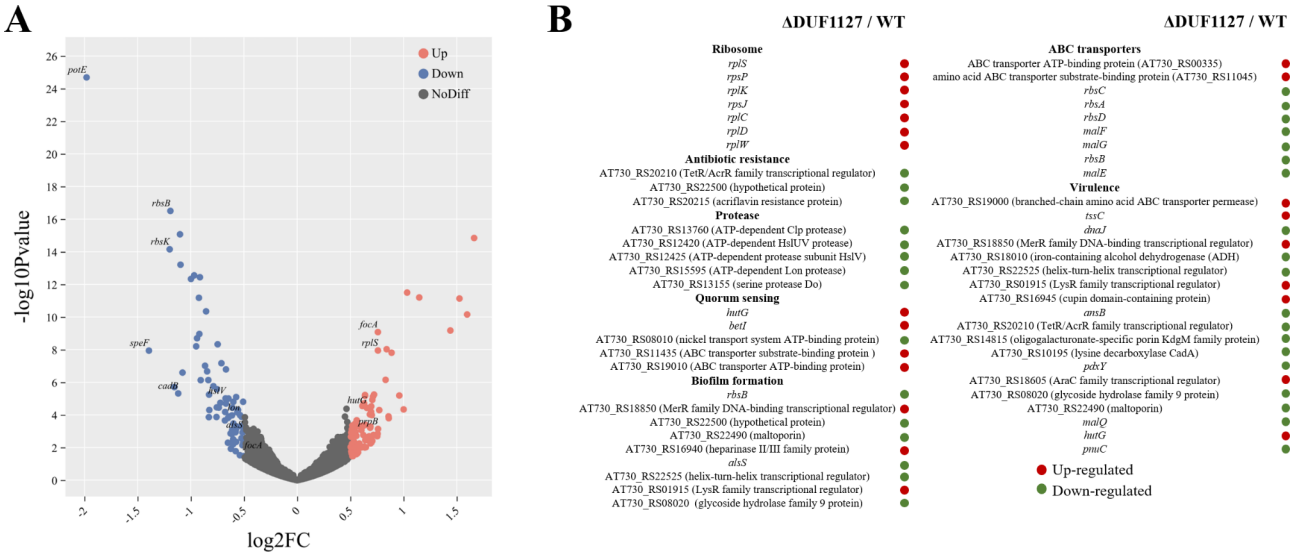

Fig. 5 Analysis of genes regulated by DUF1127 through RNA-seq. (**A**) A volcano plot presenting the differentially expressed genes in ΔDUF1127 and WT. The x-axis represents the log2 of the fold change plotted against the −log10 of the adjusted false discovery rate. Orange and blue points indicate upregulated and downregulated genes, respectively. (**B**) Map of gene expression difference. Red and green circles indicate upregulated and downregulated genes, respectively

The DUF1127-containing protein also influenced certain drug efflux-related genes, such as *vmeY* (multidrug efflux RND transporter permease subunit VmeF) and AT730_RS07965 (efflux RND transporter periplasmic adaptor subunit), moreover, the expression level of AT730 RS20215 (acriflavin resistance protein) was decreased by deleting DUF1127 (Fig. 5B, Table S3).

Moreover, ProQ regulated 218 genes, with 74 genes upregulated and 144 genes downregulated (Fig. 6A). The differentially expressed genes are involved in various processes, including biofilm synthesis (*flgC* and *flgE*) [47, 48], virulence (*betB* and *hutG*), and oxidative stress (*sodB* and *recR)* (Fig. 6B). Table S4 presents details of all differentially expressed genes. RT-qPCR was also performed to validate the RNA-seq data (Figure S6). In addition, the

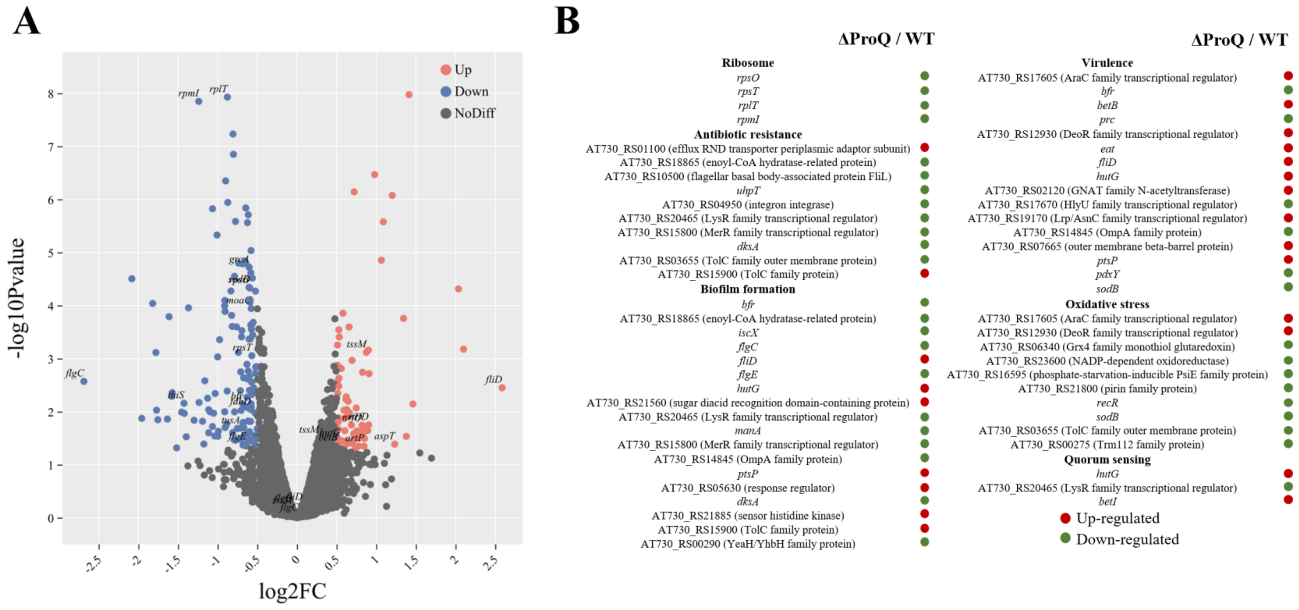

Fig. 6 Analysis of genes regulated by ProQ through RNA-seq. (**A**) A volcano plot presenting the differentially expressed genes in ΔProQ and WT. The x-axis represents the log2 of the fold change plotted against the − log10 of the adjusted false discovery rate. Orange and blue points indicate upregulated and downregulated genes, respectively. (**B**) Map of gene expression difference. Red and green circles indicate upregulated and downregulated genes, respectively

ProQ protein influenced secretion system-related genes, including AT730_RS04390 (Hcp family type VI secretion system effector), AT730_RS25955 (type IV secretion system protein), and *tssM* (type VI secretion system membrane subunit TssM) (Table S4).

The DUF1127-containing protein has been proposed as an RBP [21]. Based on the present transcriptome data, the predicted ncRNAs were filtered by length, removing those shorter than 20 bp and longer than 500 bp, and annotated using Blastx against the Nr database. Unannotated 523 sRNAs were selected as candidates for further analysis. Further analysis revealed that 32 sRNAs ($|\log_2$ fold change$| \geq 0.5$ and $P \leq 0.05$) exhibited significant differential expression, including 30 sRNAs located in the antisense to mRNA (AM) and 2 sRNAs located in the intergenic region (Table 1). Among these, 13 sRNAs were upregulated and 19 sRNAs were downregulated. Using the same method, sRNAs in the ΔProQ strain were analyzed, resulting in a total 55 sRNAs being predicted. Among them, 5 sRNAs were significantly differentially expressed, all of them were located in the AM (Table 2). The predicted sRNA Candidate_1-003–005 were significantly downregulated.

Discussion

In the present study, ΔDUF1127 had a higher stacking density of biofilm than the WT strain. Less stacking was observed in the biofilm formed by ΔProQ. Biofilm is a complex comprising proteins, extracellular polysaccharides, and DNA. Extracellular polysaccharides serve as a scaffold, and other carbohydrates, proteins, nucleic acids, and lipids adhere to this scaffold [44]. Extracellular proteins attach to the cell surface or polysaccharides, thereby aiding in biofilm formation and stability [45]. Additionally, bacteria-secreted DNA plays a crucial role in biofilm attachment [46, 47]. c-di-GMP is a widely distributed and vital second messenger in bacteria that participates in the regulation of various bacterial processes, such as biofilm formation and degradation [48, 49]. c-di-GMP is known to positively regulate biofilm formation [50, 51]. After the *proq* gene was deleted in *V. alginolyticus*, intracellular c-di-GMP level was decreased. However, in our study, after DUF1127 deletion, the c-di-GMP level decreased, but the biofilm content increased (Figure S7). The DUF1127-containing protein might affect other stronger regulatory factors than c-di-GMP that govern biofilm formation in *V. alginolyticus* [41]. Additionally, the motility of the ΔDUF1127 and ΔProQ strains exhibited no significant change, which indicated that proteins do not affect biofilm formation by influencing bacterial motility (Figure S8).

According to transcriptome analysis, the DUF1127-containing protein and ProQ affected multiple transcriptional regulatory factors (Table S3, S4). For instance, DUF1127 deletion induced the downregulation of the LysR family regulatory factors in *V. alginolyticus*. In bacteria, LysR family regulators are crucial players influencing biofilm formation. In *Klebsiella pneumoniae*, the LysR family regulator Bcal3178 positively regulates the expression of genes, including *flhD*, *phbB*, and *astB*, thus

Table 1 Predicted differentially expressed sRNAs and their target mRNAs (ΔDUF1127/WT)

Predicted sRNA	log2FC	Sequence	Predicted target mRNA	Function
sRNACandidate_2-01	1.03603	CUUUUAGAUACACUAAAAGGAACUAA	AT730_RS03370	universal stress protein UspE
sRNACandidate_2-14	-1.2719	CGCCAGACAACAAUUAAUAGUUUUAUUUAUAAUCAACGACGAUAGUAAGGCUUUAUCGUAUAAAGAGAAACGACUUUUUUAAUUUUUUAGCUCAACAUCA	AT730_RS16900	FadR family transcriptional regulator
Predicted sRNA	**log2FC**	**Sequence**	**Antisense**	**Function**
sRNACandidate_2-31	-0.5779	AUGAUUGCGAAGCCACUUUUAGGUUCGAUGUGAUUGUUGGCCAAU	AT730_RS17530	formate dehydrogenase accessory sulfurtransferase FdhD
sRNACandidate_2-02	0.73807	UGAAAUUGAACAACAAGUUGAGUCACCACUUCAUCUGA	AT730_RS18585	retention module-containing protein
sRNACandidate_2-03	0.99659	UGACCUGGUACGUCUAGAGGCAGACGCAUGAAGGCU	AT730_RS16940	oligo-alginate lyase
sRNACandidate_2-04	0.8428	AUGGCGAUAGCACAGUACCAAAC	AT730_RS17880	lipase
sRNACandidate_2-05	0.58134	UGAGCGCCAAAUAUUGCUUUUCCUCCUUUUA	AT730_RS17790	FAD-dependent oxidoreductase
sRNACandidate_2-06	0.59415	AUCUUCACAGGUAGAUUCAACAAAAUCUCAUCAGAG	AT730_RS18585	retention module-containing protein
sRNACandidate_2-07	0.8738	CCUUUUCUAGAUCGUUUCGAAAAAGCAC	AT730_RS15740	2-dehydrotetronate isomerase
sRNACandidate_2-08	0.51545	UGCAGUUUCUGCAGAGUCUUGAAUCACA	AT730_RS22550	serine/threonine transporter
sRNACandidate_2-09	0.50435	GGAAGAAAUAGGGGCUCAGCUAUUAUAUCGAACAACG	AT730_RS23185	LysR family transcriptional regulator
sRNACandidate_2-10	0.50164	AAGUUUUGUUCUGCGGUCGAAGCGAUAG	AT730_RS19430	Two-component system
sRNACandidate_2-11	0.50275	UCUGUAGUAGAACGUACCGUUGUGAGUAUCAGCAUCUCGAAUA	AT730_RS18585	retention module-containing protein
sRNACandidate_2-12	0.50328	CGAAAUGUGACCAAGCAGAUCAUCCAUGUCGAUCAUUGAGUUAUUGAGAUCAGAGA	AT730_RS18585	retention module-containing protein
sRNACandidate_1-13	0.51842	CCACUUUUUACUUUACCAUCGAUGUA	AT730_RS12975	mannitol PTS system EIICBA or EIICB component
sRNACandidate_1-16	-3.6341	GCGAAUAUAUUCAUUUGGACAUCU	AT730_RS10195	lysine decarboxylase
sRNACandidate_2-15	-1.2173	CCCGGCCAGUCUCGAUUUCUACAAAGAAGUGG	AT730_RS22505	glycosidase
sRNACandidate_1-17	-0.7302	CAGUAUUUCUCUGGAUUCGUUUCGUGUCGCUGGC	AT730_RS06365	multiple antibiotic resistance protein
sRNACandidate_2-18	-1.0441	CCAAUAAAGGUCAUCGCCCAAUCACUGCUAUCAGACCAC	AT730_RS22515	carbohydrate metabolic process
sRNACandidate_1-19	-0.678	UAGCGUCAUACUCGCGCCCGUCAAAGAG	AT730_RS13155	serine protease Do
sRNACandidate_2-20	-0.7203	UUUGUUGUCAUUAUUAUUACUGCUUAAUAAAUACUGCUUGUCAGUUUGUUAGCUUAAU	AT730_RS23410	DUF58 domain-containing protein
sRNACandidate_2-21	-0.786	UUUUUCGUUCCUUACGCAGC	AT730_RS11165	Bcr/CflA family multidrug efflux MFS transporter
sRNACandidate_1-22	-0.5794	GAAAUCGUUCCAGACAAGACAGUGCA	AT730_RS13760	ATP-dependent Clp protease ATP-binding subunit
sRNACandidate_1-23	-0.5211	UCUUCUUUGCCAUCAGGGUAU	AT730_RS00175	DUF1887 family protein
sRNACandidate_1-24	-0.5117	UCGCAAUAUUCUUCAUCUGA	AT730_RS04645	DUF4123 domain-containing protein
sRNACandidate_1-25	-0.5308	UGGGGAACAGAUGUUAAUUCUAUCCCAACGCGC	AT730_RS12260	L-malate glycosyltransferase
sRNACandidate_1-26	-0.5765	AAGCUGAAGCACAAGAAGUUGAAGCUGAGCUUGAAGAGCUUGGUGAUGAGACAGACGCUAAGAUUGCUCAACUAGAAGCGGC	AT730_RS14225	molecular chaperone GrpE
sRNACandidate_1-27	-0.5921	UUCAUGGCUAUUGUUCCGCUCUGC	AT730_RS06365	multiple antibiotic resistance protein
sRNACandidate_1-28	-0.5685	GUUUAUGGUGCACGCUACCCAAG	AT730_RS06365	multiple antibiotic resistance protein
sRNACandidate_2-29	-0.6427	UCGCGAGCACUUUCUACUGCUUCUAGUCGACGGAACUCAGGAU	AT730_RS23395	Ca-activated chloride channel homolog
sRNACandidate_2-30	-0.6174	CGUCAUUUUGUAAAUUUGUAAUAGCACCAUUAU	AT730_RS19040	6-phospho-alpha-glucosidase
sRNACandidate_1-32	-0.5543	AGUGCUUCUAGCUCAACACG	AT730_RS12420	HslU--HslV peptidase ATPase subunit

promoting bacterial biofilm synthesis [49]. The STM0859 protein with a DNA-binding domain is a transcriptional LysR family regulator and promotes biofilm formation in *S. typhimurium* by binding to the *rcsb* promoter fragment [49]. Moreover, *alsS* is involved in cell lysis and eDNA release, and downregulation of *alsS* expression may lead to more biofilm formation in the DUF1127 deletion strain [46]. The target genes bound by these differentially

Table 2 Predicted differentially expressed sRNAs and their target mRNAs (ΔProQ/WT)

Predicted sRNA	log2FC	Sequence	Antisense	Function
sRNACandidate_1-001	0.935346	CUACCAAUUCCGCCACUUCCGCAACUUA	AT730_RS14675	tRNA-Leu
sRNACandidate_1-002	0.906888	GCCCUACAUUGCUCUAAUAA	AT730_RS14670	tRNA-Gln
sRNACandidate_1-003	-0.55338	GCCUGCACGGAUAGUGUUAAC	AT730_RS12765	TAXI family TRAP transporter solute-binding subunit
sRNACandidate_1-004	-0.59618	GUUGAAAGGUUCUGUAUGAAGAGAGAAC	AT730_RS12765	TAXI family TRAP transporter solute-binding subunit
sRNACandidate_1-005	-1.0166	AGUGGGCAGUGGCUUCACCUUCAAC	*recR*	Recombination mediator RecR

expressed sRNAs in the DUF1127 deletion strain participate in various aspects, including regulation, protein secretion, and metabolism. In the transcriptome, *grpE* was significantly downregulated, and its complementing sRNA (sRNACandidate_1–26) was also significantly downregulated. This implied that DUF1127 may influence *grpE* by downregulating sRNACandidate_1–26, subsequently regulating biofilm synthesis in *V. alginolyticus* [57].

The phosphomannose isomerase-encoding gene, *manA*, was significantly downregulated after *proq* was deleted (Fig. 6). *manA* is crucial for producing extracellular polysaccharides, a essential component in the biofilm matrix. High c-di-GMP concentrations inhibit the binding of the Clp transcription factor to *manA*'s promoter, thereby suppressing *manA* expression [50]. After *proq* was deleted, the *ompA* transcription level was significantly downregulated (Fig. 6). The outer membrane protein OmpA is a crucial component of *V. alginolyticus* biofilms. *ompA* deletion reduced the biofilm synthesis capacity of *V. alginolyticus* [51, 52].

ProQ assisted bacteria in resisting oxidative stress. According to the transcriptome analysis of the ProQ deletion strain, the transcription levels of *recR* (recombination mediator RecR) and *sodB* (superoxide dismutase (SOD)) were significantly downregulated (Fig. 6). In *Helicobacter pylori*, RecR is essential for resistance to oxidative stress in bacteria. The *recR* mutant strain exhibited significantly reduced survival in ambient air compared with the WT strain [53]. SOD is a widely distributed antioxidant defense metal enzyme that catalyzes the of superoxide anions into oxygen and hydrogen peroxide. This enzyme thus protects cells from the damaging effects of the anions. ProQ may assist *V. alginolyticus* in resisting oxidative stress by upregulating *sodB* and *recR* expression. Rockhopper software analysis unveiled that the target gene of sRNACandidate_1–005 is *recR*. Therefore, we hypothesize that ProQ indirectly regulates *recR* by positively controlling sRNACandidate_1–005, thereby aiding *V. alginolyticus* in oxidative stress resistance.

A TetR-type transcriptional regulatory factor, BetI, was significantly upregulated after DUF1127 and ProQ were deleted (Figs. 5, 6). The TetR family regulatory factors bind to the promoters of efflux pump genes, thereby affecting bacterial resistance to antibiotics [54]. Therefore, antibiotic resistance of *V. alginolyticus* was also assessed. DUF1127 and ProQ proteins were found to influence the antibiotic resistance of *V. alginolyticus* (Figure S9).

Various proposed sRNAs have showed change expression level by deleting DUF1127-containing protein. In *Rhodobacter sphaeroides*, DUF1127 protein CcaF1 recognition element contains a stem–loop structure with the sequence CUGGC in the loop [21]. The similar structure and sequence were also found in sRNACandidate 1–17 (Figure S10). The prediction of sRNAs regulated by ProQ using the same method yielded limited results, may due to the inappropriate timing of RNA extraction. Certain sRNAs may be expressed during specific growth stages or under particular environmental conditions, while being scarce or inactive under other conditions [55]. Subsequent efforts could involve processing samples from multiple time points to enhance the prediction of sRNAs regulated by ProQ. Moreover, RIP will be performed to get information about sRNAs which can directly bind to DUF1127-containing protein and ProQ.

Conclusion

We here showed that the RBP DUF1127-containing protein exerts a negative regulatory effect on biofilm formation in *V. alginolyticus*. By contrast, ProQ positively regulates biofilm formation. Moreover, DUF1127 increases the level of extracellular proteinase secreted, and ProQ deletion renders cells sensitive to oxidative stress. The regulation mechanisms of the DUF1127-containing protein and ProQ in *V. alginolyticus* needs to be further studied.

Abbreviations

RBPs RNA-binding proteins
sRNAs Small RNAs

Supplementary Information

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Supplementary Material 1

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Author contributions

Ruonan Feng and Ying Chen wrote the main manuscript text and supplement. Ruonan Feng and Tao Peng prepared Figs. 1, 2, 3, 4, 5 and 6. Ying Chen and

Tongxian Chen prepared the Table 1 and 2. Tao Peng and Zhong Hu designed the work. Tao Peng revised the manuscript.

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Data availability
The sequencing data was uploaded to SRA (NCBI) under the accession number PRJNA962541 and PRJNA1066051.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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