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Original Article

Conditional gene silencing in the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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ABSTRACT

Since the release in 2005 of the genome sequence and annotation of the first Antarctic marine bacterium, the number of genomes of psychrophilic microorganisms in public databases has steadily increased. Unfortunately, the lack of effective molecular tools for the manipulation of these environmental strains still hampers our understanding of their peculiar strategies to thrive in freezing conditions, limiting the functional genomics approaches to differential analyses only. Over the past two decades, our research group established the first effective gene cloning/expression technology in the Antarctic Gram-negative marine bacterium *Pseudoalteromonas haloplanktis* TAC125. The setup of a genome mutagenesis technique (based on homologous recombination and counterselection events) further supported the use of this strain, which became an attractive model for studying microbial adaptations to freezing lifestyle. Moreover, to further extend the functional analyses to its essential genes, the set-up of a conditional gene silencing approach is desirable. In this paper, we report the development of an asRNA regulatory system in the Antarctic bacterium, testing the feasibility of Hfq-dependent and PTasRNA strategies previously developed in *Escherichia coli*. Stable and efficient silencing of two chromosomal genes was obtained by using PTasRNAs, reaching very high levels of downregulation.

1. Introduction

Small RNAs (sRNAs) are regulatory RNAs involved in controlling gene expression at the post-transcriptional and/or translational level by base-pairing with their target mRNA [1]. Given the typical complementarity of their sequence with their targets, they are often denoted as antisense RNAs (asRNAs). Most bacterial asRNAs act by binding to the translation initiation region (TIR) of the transcripts, so as to interfere with the efficiency of ribosome recruiting. As a result, the translation is hindered, and the target mRNA is destabilized and susceptible to degradation [2]. Furthermore, many asRNAs can act against multiple targets explaining why they are involved in various processes such as stress responses, metabolic switches, virulence, biofilm formation, and much more [1].

In recent years, the repurposing of natural asRNAs to develop efficient gene regulation tools has widely spread. Indeed, several technologies were established in bacteria, based on engineered asRNAs, such as synthetic asRNA [3], CRISPR interference (CRISPRi) [4], CRISPR activation (CRISPRa) [5], small transcription activating RNA (STAR) [6] and riboswitch [7]. Relying on the Watson-Crick base pairing principle, such strategies hold many advantages compared to DNA or protein-based methods. They allow easy, predictable, scalable, and finely tunable control of multiple genes — even essential ones [8] — for very diverse applications, spanning from physiological studies to metabolic engineering and therapeutics [9,10].

Na and coworkers reported a strategy to design synthetic asRNAs in *Escherichia coli* (*E. coli*) with high repression capability (>90%) [3]. They identified MicC as one of the asRNA able to efficiently interact with the RNA chaperone protein Hfq in *E. coli* and developed an Hfq-dependent asRNA by the functional modularization of MicC sequence into a target binding region and a scaffold that recruits Hfq. Even if the gene silencing is strictly exerted by the target binding sequence and is strongly dependent on the efficiency

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of TIR masking, the interaction of synthetic asRNA with Hfq ensures a more efficient outcome.

Nakashima and collaborators designed another RNA-based regulatory tool in *E. coli*, known as Paired Termini antisense RNAs (PTasRNAs). A PTasRNA consists of a hairpin structure whose loop holds the antisense sequence able to bind the RBS of the target mRNA [11]. The complementarity of the terminal regions positively affects PTasRNAs stability, consequently enhancing their cellular concentration and silencing efficacy compared to canonical asRNA. PTasRNAs turned out to be quite successful in regulating multiple essential genes in *E. coli* [12].

Notably, the versatility and ease of such approaches can also be extended to less characterized and almost "wild" environmental microorganisms, often tricky to handle in routine genetic manipulations, given the often observed ineffectiveness of conventional mutagenesis techniques, due to the low efficiency of genome integration of exogenous DNA through homologous recombination [13–15].

In this respect, from the release of the genome sequence and annotation of the first Antarctic marine bacterium in 2005 [16], the number of genomes of psychrophilic microorganisms in public databases has steadily increased. On the contrary, the setup of molecular tools available for the manipulation of these environmental strains did not follow the same trend, limiting genetic interventions to few genera only. Among them, *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) [16], also named *Pseudoalteromonas translucida* TAC125, was the first Antarctic Gram-negative bacterium in which an effective gene cloning/expression technology was established [17,18], becoming a robust model for studying microbial adaptations to freezing lifestyle [19–21]. Although a genome mutagenesis technique was established in *Ph*TAC125 by our group [22], it relies on homologous recombination and counterselection events, often occurring at a frequency only slightly higher than spontaneous mutations. Moreover, the set-up of a conditional gene silencing approach in the psychrophilic bacterium would significantly widen the tools for its gene functional analysis, as the gene insertion/deletion technology is not applicable to essential genes.

In this work, Hfq-dependent and PTasRNA strategies were applied to the silencing of the *Ph*TAC125 gene coding for the Lon protease. This gene was chosen because the commercial antibodies raised against the *E. coli* Lon protease efficiently recognize the psychrophilic protease, owing to their high sequence similarity, allowing the easy evaluation of the silencing effects at the protein level by quantitative Western blotting. Furthermore, Lon is a popular target for the improvement of many common hosts for recombinant protein production, as it is reported as the main protease involved in the degradation of intracellular proteins [23,24]. The PTasRNA technology gave back the more efficient and durable silencing results, and it was applied to the downregulation of a less-intensively transcribed *Ph*TAC125 gene (*PhhbO*). It encodes a truncated hemoglobin (trHbO) involved in the cellular protection against oxidative stress [25]. The phenotypic characterization of the interfered strain demonstrated that *PhhbO* expression was efficiently repressed by the PTasRNA strategy, extending the design principles of these synthetic asRNAs to the application in psychrophilic bacteria.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli DH5α [*supE44, ΔlacU169 (φ80 lacZΔM15) hsdR17, recA1, endA1, gyrA96, thi-1, relA1]* was used for cloning procedures. *E. coli* S17-1 (λ*pir*) *[thi, pro, hsd (r− m+) recA::RP4-2-TCr::Mu Kmr::Tn7, Tpr, Smr, λpir]* [26] constituted the donor strain in intergeneric conjugations for KrPL transformations [17,27]. KrPL – a *Ph*TAC125 strain cured of its endogenous pMtBL plasmid [27] – was used as the host for expressing antisense RNAs throughout the study. Both *E. coli* strains were grown in LB broth (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 34 μg/mL chloramphenicol at 37 °C. KrPL was grown in TYP (16 g/L bacto-tryptone, 16 g/L yeast extract, 10 g/L NaCl) during conjugation experiments and in GG (10 g/L L-glutamic acid monosodium salt monohydrate, 10 g/L D-gluconic acid sodium salt, 10 g/L NaCl, 1 g/L NH_4NO_3, 1 g/L K_2HPO_4, 200 mg/L $MgSO_4 \cdot 7H_2O$, 5 mg/L $FeSO_4 \cdot 7H_2O$, 5 mg/L $CaCl_2$) [21] in recombinant production experiments. Recombinant KrPL was grown in solid media supplemented with 12.5 μg/mL chloramphenicol, while in liquid media the antibiotic was added at 25 μg/mL.

2.2. Construction of expression plasmids

The nucleotide sequences of synthetic asRNAs and oligonucleotide primers used in this work are reported in Table 1.

The DNA fragment containing the antisense sequence targeting *lon* mRNA, MicC scaffold and T1/TE terminator was synthesized by Thermo Fisher Scientific and cloned into pB40-79C, a high-copy number derivative of p79C [27] (unpublished data), using NheI and SacI restriction sites. The resulting construct was pB40-79C-asRNA-*lon* (Fig. S1A).

For the construction of pB40-79C-PTasRNA-*lon* and pB40-79C-5′-PTasRNA-*lon,* the intermediate plasmid pB40-79BsC was developed. pB40-79BsC differs from pB40-79C for the removal of HindIII, EcoRI, NheI and NdeI restriction sites present in the divergent *lacR* gene and P*lacZ* promoter, and for the introduction of BsaI and PstI sites immediately downstream of the P*lacZ* promoter (Fig. S1B). To obtain this plasmid, a synthetic fragment (Thermo Fisher Scientific) containing the *lacR* gene and P*lacZ* promoter with the above-mentioned modifications was digested in SphI and PstI sites and cloned into a high-copy number derivative of pUCC [28] (unpublished data) hydrolyzed with the same enzymes. Then, the synthetic DNA fragments (Thermo Fisher Scientific) containing PTasRNA-*lon* and 5′-PTasRNA-*lon* sequences were cloned into pB40-79BsC using BsaI and SmaI restriction sites (Fig. S1C).

For the construction of pB40-79C-PTasRNA2-*lon* and pB40-79C-PTasRNA3-*lon*, two DNA fragments of the *lon* gene (*PSHA_RS10175*) were amplified by PCR using the PT2_fw- PT2_rv and PT3_fw-PT3_rv primer pairs, respectively. The reaction was performed using 1 unit Phusion DNA Polymerase (New England Biolabs), 0.5 μM each primer, 200 μM dNTP, 1X HF Phusion buffer, and 50 ng *Ph*TAC125 genomic DNA as the template. The thermocycling condition was set up as follows: one cycle at 98 °C for 30 s, 25 cycles at 98 °C for 10 s, 67 °C for 30 s, 72 °C for 15 s, followed by the last cycle at 72 °C for 5 min. Then, the obtained amplicons were digested with PstI and XhoI and cloned into pB40-79C-PTasRNA-*lon* hydrolyzed with the same restriction enzymes (Fig. S1D).

pB40-79C-PTasRNA-*hbO* was constructed with the same strategy. The DNA fragment containing the TIR of *hbO* (*PSHA_RS00150*) was obtained through a PCR amplification by using hbO_fw-hbO_rv primers. The reaction was performed using 1 unit Phusion DNA Polymerase (New England Biolabs), 0.5 μM each primer, 200 μM dNTP, 1X HF Phusion buffer, and 50 ng *Ph*TAC125 genomic DNA as the template. The thermocycling condition was set up as follows: one cycle at 98 °C for 30 s, 20 cycles at 98 °C for 10 s, 71 °C for 30 s, 72 °C for 15 s, followed by the last cycle at 72 °C for 5 min. Then, pB40-79C-PTasRNA-*lon* and the amplicon were digested with PstI and XhoI and ligated (Fig. S1D).

The sequence of all PCR-amplified fragments was checked to rule out the occurrence of unwanted mutations.

Table 1
Nucleotide sequences of synthetic asRNAs and oligonucleotide primers used in this work. Fw: forward; Rv: reverse.

asRNA	Sequence (5' - 3')
asRNA-*lon*	TCGATCGGTTCTCTCAAGCGTCATTTTC TGTTGGGCCATTGCATTGCCACTGATTTT CCAACATATAAAAAGACAAGCCCGAACAG TCGTCCGGGCTTTTTTTCTCGAGCCAGGC ATCAAATAAAACGAAAGGCTCAGTCGAAA GACTGGGCCTTTCGTTTTATCTGTTTTTGT CGGTGAACGCTCTCTACTAGAGTCACACTG GCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA
5′-PTasRNA-*lon*	TATAATTGCCCGTTTATGCAACAGGAATAAAC AGGAGGAATTAACCATGCAGTGGTGGTGGTG GTGGTGCTGCAGGCGGGTATACCACTACATC GCGCAGTGCTAATACTGGGATTTCGACTCGA TCGGTTCTCTCAAGCGTCATTATATTTCTCTT TGCACTTCATTTATTTTAAAGATTACTCCTCG AGCACCACCACCACCACCACTGCATGGTTA ATTCCTCCTCCCGGG
PTasRNA1-*lon*	AGGAGGAATTAACCATGCAGTGGTGGTGGTG GTGGTGCTGCAGGCGGGTATACCACTACATC GCGCAGTGCTAATACTGGGATTTCGACTCGAT CGGTTCTCTCAAGCGTCATTATATTTCTCTTG CACTTCATTTATTTTAAAGATTACTCCTCGAGC ACCACCACCACCACCACTGCATGGTTAATTCC TCCTCCCGGG
PTasRNA2-*lon*	AGGAGGAATTAACCATGCAGTGGTGGTGGTGG TGGTGCTGCAGCATGTGCGGGTATACCACTAC ATCGCGCAGTGCTAATACTGGGATTTCGACTC GATCGGTTCTCTCAAGCGTCATTATATTTCTCT TTGCACTTCATTTATTTTAAAGATCTCGAGCAC CACCACCACCACCACTGCATGGTTAATTCCTC CTCCCGGG
PTasRNA3-*lon*	AGGAGGAATTAACCATGCAGTGGTGGTGGTG GTGGTGCTGCAGTATACCACTACATCGCGCA GTGCTAATACTGGGATTTCGACTCGATCGGTT CTCTCAAGCGTCATTATATTTCTCTTTGCACTT CATTTATTTTAAAGATTACTCAAGTACTCGAG CACCACCACCACCACCACTGCATGGTTAATT CCTCCTCCCGGG
PTasRNA-*hbO*	AGGAGGAATTAACCATGCAGTGGTGGTGGTG GTGGTGCTGCAGGTGTTTTTTCAGGTGTGGGT GTTTGCTCTATTGTGGCGGGCTTAGATTTTGA AAAAAGTCGTTTAATCATATTATTTATCATCG TTAATAGGCAGTATTACTACTGCAAACTCGAG CACCACCACCACCACCACTGCATGGTTAATT CCTCCTCCCGGG

Primer	Sequence (5' – 3')
PT2_fw	CAGGCTCGAGATCTTTAAAATAAATGAAGTGCAAA
PT2_rv	CGACTGCAGCATGTGCGGGTATACCACTAC
PT3_fw	CAGGCTCGAGTACTTGAGTAATCTTTAAAATAAAT
PT3_rv	CGACTGCAGTATACCACTACATCGCGC
hbO_fw	CAGGCTCGAGTTTGCAGTAGTAATACTGCCTATTA
hbO_rv	CGACTGCAGGTGTTTTTTCAGGTGTGGG

2.3. Recombinant production procedures

Glycerol stocks (- 80 °C) of KrPL recombinant strains were streaked over TYP agar plates and incubated at 15 °C for 72 h. Then, a single colony was inoculated in 2 mL of TYP at 15 °C for 1–2 days. After a first adaptation step, performed by diluting 100-fold the culture in GG medium, the inoculum was performed at 0.1 OD/mL. The recombinant production was performed at 15 °C and the induction was carried out with 10 mM IPTG when the cells reached the middle-late exponential growth phase. A Biosan PSU-20i orbital shaker was used setting the agitation at 180 rpm.

2.4. SDS-PAGE and western blotting

To quantify Lon protease intracellular levels, cell pellets corresponding to the number of cells yielding an absorbance of 1 OD_{600} ml^{-1}, were collected by centrifugation at 11,000×*g* at 4 °C and solubilized in 60 μL of Laemmli buffer 4X. Then, the samples were boiled at 95 °C for 20 min and centrifuged at 10,000×*g* for 1 min at RT. 3 μL of samples were run on a TGX Stain-Free Mini-Protean 4–15% (BioRad) gel in TGS buffer for 90 min at 110 V. The gel was then Stain-Free activated for 2.5 min and imaged using the ChemiDoc MP imaging system (BioRad) and ImageLab software (version 6.0, BioRad). Then, the separated proteins were transferred to an Immun-Blot low-fluorescence polyvinylidene fluoride (PVDF) membrane (BioRad) in 7 min using the TransBlot Turbo Transfer System (BioRad) with the mixed molecular weight setting. After the transfer, the membrane was again imaged and blocked with PBS, 0.05% Triton X-100, 5% (*w/v*) milk for 1 h. Then, an anti-*Ec*Lon antibody (ab103809) was diluted 1: 5000 in the same buffer. After 1 h of incubation at RT with the primary antibody, the membrane was washed with PBS, 0.05% Triton X-100 three times (5 min each) and incubated with an HRP-conjugated anti-rabbit antibody diluted 1:3000 in PBS, 0.05% Triton X-100, 5% (*w/v*) milk for 1 h at RT. Then, the membrane was washed again with PBS, 0.05% Triton X-100 three times (5 min each) and the secondary antibody was detected using the ECL method (Cyanagen).

2.5. Western blotting data analysis

The signal intensities of Lon in each lane were determined by using the "Lane and Bands" tool of ImageLab software (version 6.0, BioRad). The densitometric analysis was performed by normalizing bands to total proteins in each lane detected on the blot membrane as previously described [29].

2.6. Bioinformatics analysis

The prediction of RNA secondary structures was performed using mFold website with default settings [30]. RNAPredator [31] was used for the prediction of asRNA-mRNA interaction setting NC_007481 for the selection of the *Ph*TAC125 genome.

2.7. H_2O_2 disk-inhibition assay

Cultures of psychrophilic non-recombinant cells, and those harboring either the pB40-79C-PTasRNA-*hbO* vector or the empty vector pB40-79BsC were diluted in 7.5 mL warm TYP soft agar (4 g/L agar) at a final concentration of 0.2 OD/mL. When required, 12.5 μg/mL chloramphenicol and 10 mM IPTG were added to the media. Then, a disk of Whatman filter paper was soaked in 256 mM H_2O_2 and placed in the center of each plate. After 24 h of incubation at 15 °C, the diameter of the killing zone was measured. Data deriving from two independent experiments, each carried out in quintuplicate, were used to calculate data reported in Table 3.

2.8. Statistics and reproducibility of results

Data were statistically validated using the t-Student test comparing the mean measurements of silenced and non-silenced samples. The significance of differences between mean values was calculated using a two-tailed Student's t-test. A P value of <0.05 was considered significant.

3. Results

3.1. Construction of Hfq-dependent asRNA-Lon and evaluation of Phlon silencing efficiency in Ph*TAC125*.

Following the established design criteria (as described in [3]), the asRNA-*lon* was obtained by fusing the Hfq-binding scaffold

Table 2
Prediction of the interactions occurring between PTasRNAs and their target *lon* mRNA in *Ph*TAC125. RNAPredator tool was used to predict the total binding energy, the corresponding Z-score, the coordinates on asRNA and mRNA. Each prediction is presented with the experimental results achieved in *Ph*TAC125 expressed as maximum silencing activity.

PTasRNA-mRNA binding energy						
Variant	Energy [kJ/mol]	z-Score	asRNA	mRNA [Start]	mRNA [End]	Maximum silencing activity
PTasRNA1-lon	−41.19	−8.05	95–124	−9	20	70%
PTasRNA2-lon	−39.47	−7.57	105–134	−14	15	54%
PTasRNA3-lon	−35.20	−9.92	44–73	37	66	47%

Table 3
Diameter of the disk-inhibition zone. Bacterial growth sensitivity to H_2O_2 is directly proportional to the diameter of the disk-inhibition zone. IND, The given recombinant strain was grown and tested in the presence of 10 mM IPTG to induce the transcription of PTasRNA-*hbO* or the void vector. The reported values represented the mean of two independent experiments, each carried out in quintuplicate, and were considered significant when $p < 0.001$ according to the t-Student test.

Strain	Diameter of the disk-inhibition zone (cm)
*Ph*TAC125 wt	0.16 ± 0.04
*Ph*TAC125-30mut	1.24 ± 0.09
KRPL-pB40-79BsC IND	0.28 ± 0.06
KRPL-pB40-79C-PTasRNA-*hbO* IND	1.32 ± 0.08

derived from *E. coli* MicC asRNA with a sequence complementary to the first 24 bp of the TIR of the *Ph*Lon mRNA (*PSHA_RS10175*) (see Table 1). The synthetic asRNA and the target mRNA are expected to interact with an energy of hybridization that can be predicted using RNAPredator tool. The expected hybridization energy of asRNA-*lon* with its target turned out to be −42 kcal mol^{-1}, placing the interaction within the optimal binding energy range from −30 to −40 kcal mol^{-1} [3]. Finally, a strong transcription terminator T1/TE was also added to guarantee the release of the nascent asRNA transcript (Fig. 1A, B).

Once designed, asRNA-*lon* was cloned into pB40-79C, a high copy number variant of p79C [27] (unpublished data). Then, the vector was transferred into KrPL, a *Ph*TAC125 strain devoid of the endogenous pMtBL plasmid [27], without any noticeable effect on the growth behavior of the recombinant cells (Fig. S2). The recombinant expression of Hfq-dependent asRNA-*lon* was performed as described in the Material and Methods section. The induction was performed during the exponential growth phase and the occurrence of gene silencing was evaluated by quantitative Western blot. In particular, the analysis was performed on samples collected 2, 4, 8, 24 and 32 h after the induction by measuring Lon levels detected in total protein extracts of cells expressing the asRNA-*lon* in comparison to cells carrying the empty vector and collected at the same time point post induction (NC). The amount of Lon protease detected in any given NC sample was set to unit and used to normalize the amount detected in the silenced cells (Fig. 1C). A statistically relevant reduction in Lon concentration was recorded only 2 h post-induction. At the following time points, the protease was consistently detected in almost comparable quantities in the two strains.

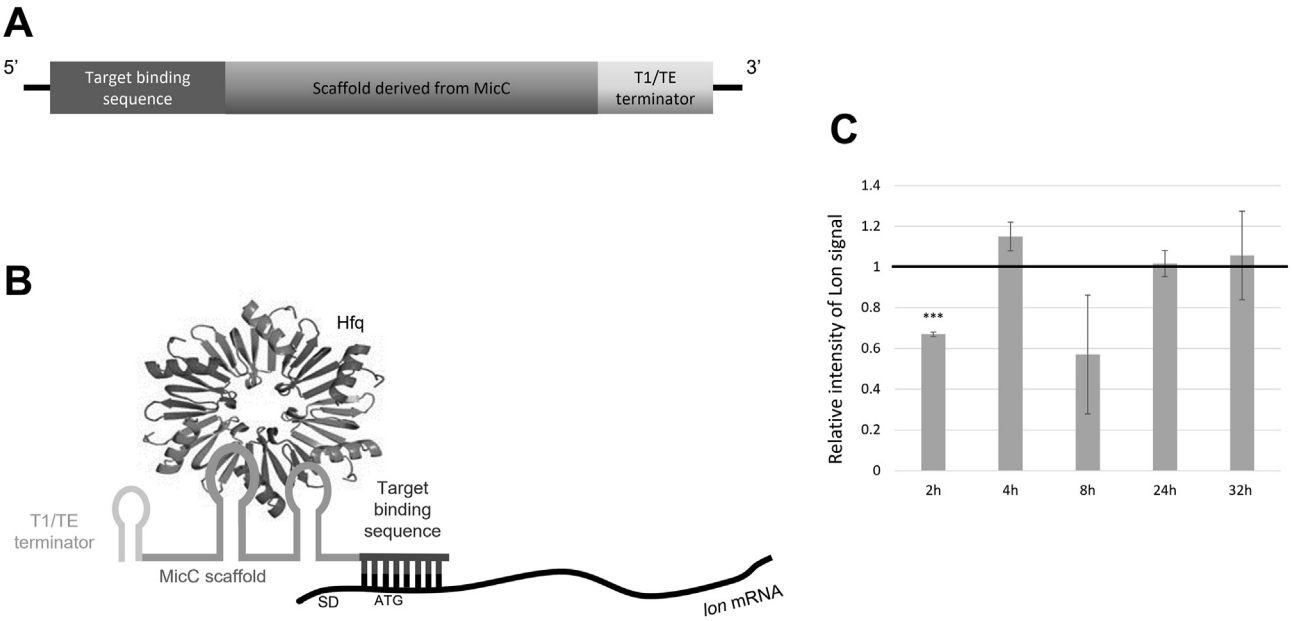

Fig. 1. Evaluation of *Phlon* gene silencing mediated by Hfq-dependent asRNA-*lon*. **(A)** Schematic representation of Hfq-dependent asRNA-*lon* construct. A sequence complementary to the TIR of *lon* mRNA was fused to the sequence of *E. coli* MicC recruiting Hfq proteins. To ensure the termination of the transcription, a synthetic T1/TE terminator was added. **(B)** Suggested mode of action of Hfq-dependent asRNA-*lon*. The antisense sequence binds the Transcription Iniziation Region (TIR) of *lon* mRNA, downstream of the Shine-Dalgarno (SD) sequence, sequestering the transcript from the ribosomes. In this way, it acts by repressing the translation. The Hfq protein binds to the MicC derived scaffold, aiding the formation of the asRNA-mRNA complex resulting in a more efficient gene silencing. **(C)**. Evaluation of relative concentration of Lon protease in cells expressing Hfq-dependent asRNA-*lon* compared to control cells, carrying the empty vector. Western blotting was performed on total protein extracts of cells recovered 2, 4, 8, 24 and 32 h after the induction of asRNA-*lon* transcription using anti-*Ec*Lon antibodies. The black bar represents the baseline amount of Lon detected in the control samples. The measurements are reported as the mean of two independent experiments whose standard deviation is indicated by the error bars. The data were considered significant when $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) according to t-Student test.

3.2. Construction of PTasRNAs and evaluation of Phlon silencing efficiency in Ph*TAC125*

A second approach to achieve an efficient *Phlon* gene silencing was performed by using Paired Termini antisense RNAs (PTasRNAs). These molecules contain 38 nt-long terminal inverted repeats, characterized by a high GC content, and allowed a high silencing efficiency in *E. coli* without triggering any interference on plasmid stability [11]. mFold secondary structure prediction tool was used to arrange the antisense sequence (110 nt), harboring the *Phlon* RBS and start codon, within a loop region easily accessible for the hybridization with the target mRNA (Fig. 2A, B). To explore the effect of the specific binding region on TIR sequestering, three variants were designed, each characterized by a shift of 5 nt of the antisense sequence towards *Phlon* 3′-end, resulting in PTasRNA3-*lon,* PTasRNA1-*lon,* and PTasRNA2-*lon*, respectively (Fig. 3A). The constructs were cloned into the psychrophilic expression vector pB40-79BsC and expressed in KrPL without any noticeable effect on the growth behavior of the recombinant cells (Fig. S3A).

As previously described changes in Lon protease concentration in cells expressing the different PTasRNAs were evaluated through quantitative Western blot analysis (Fig. 3B). Again, total protein extracts of KrPL cells bearing the empty vector and subjected to the same induction protocol were used to assess the normal Lon amount in non-silenced cells. PTasRNA1-*lon* markedly reduced the concentration of Lon by about 70% after 8 h of induction, and the effect lasted up to 32 h. Both variants PTasRNA2-*lon* and PTasRNA3-*lon* achieved lower silencing efficacy than the first version, with a maximum activity of about 54% and 47%, respectively. Furthermore, they showed a less durable effect since the repression began to wane after 24 h post-induction.

Interestingly, a direct correlation between repression efficiency and interaction binding energy predicted by RNApredator software was observed (Table 2). This finding suggested that a strong interaction is required for satisfying target suppression also in psychrophilic bacteria.

For the reasons stated above, PTasRNA1-*lon* was selected for further experiments.

As described by Nakashima and coworkers, the presence of a 5′ leader sequence upstream to the hairpin structure may exert a stabilizer effect on the synthetic RNA half-life [11], potentially enhancing its repression efficiency. Hence, we designed a variant of PTasRNA1-*lon,* called 5′-PTasRNA1-*lon*, differing just for the presence of the extremely stable 5′-UTR from *PhlacZ* mRNA [27]. This asRNA was constructed and cloned into pB40-79BsC (Fig. 4A), and its recombinant production was then performed in KrPL cells as described above.

Once confirmed that the overexpression of PTasRNAs did not induce either any plasmid instability or non-specific silencing of other essential genes (Fig. S3B), their silencing capability was analyzed by quantitative Western blotting (Fig. 4B). The densitometric analysis revealed that 5′-PTasRNA1-*lon* is less effective than PTasRNA1-*lon* during the first 8 h of expression. Then, the two constructs exerted a comparable effect with about 70% reduction of Lon levels after 24 h of induction.

These results suggested that the 5′-UTR sequence did not contribute to the enhancement of the asRNA repression capability.

3.3. Validation of PTasRNA technology in PhTAC125 by silencing PhHbO encoding gene

The design of PTasRNA-*hbO* was performed considering the results obtained with the *lon* target. In detail, the synthetic asRNA was made up by the already used 38 bp long terminal inverted repeats, while the 110 nt long antisense sequence was selected following the predictions of RNAPredator tool. The sequence potentially binding to the TIR elements of *PhhbO* mRNA with the highest estimated efficiency (binding energy of −36.81 kJ/mol), and negligible non-specific off-target binding, was PCR amplified. Then, the gene coding the assembled PTasRNA-*hbO* was cloned into the psychrophilic expression vector pB40-79BsC and recombinantly expressed in KrPL. Since the *Ph*TAC125 truncated hemoglobin trHbO displays a notable peroxidase activity [25], the occurrence of the conditional control of its mRNA translation was evaluated by a disk-diffusion assay in the presence of 256 mM H_2O_2 (Fig. 5). As expected, *Ph*TAC125 wild type cells and recombinant KrPL

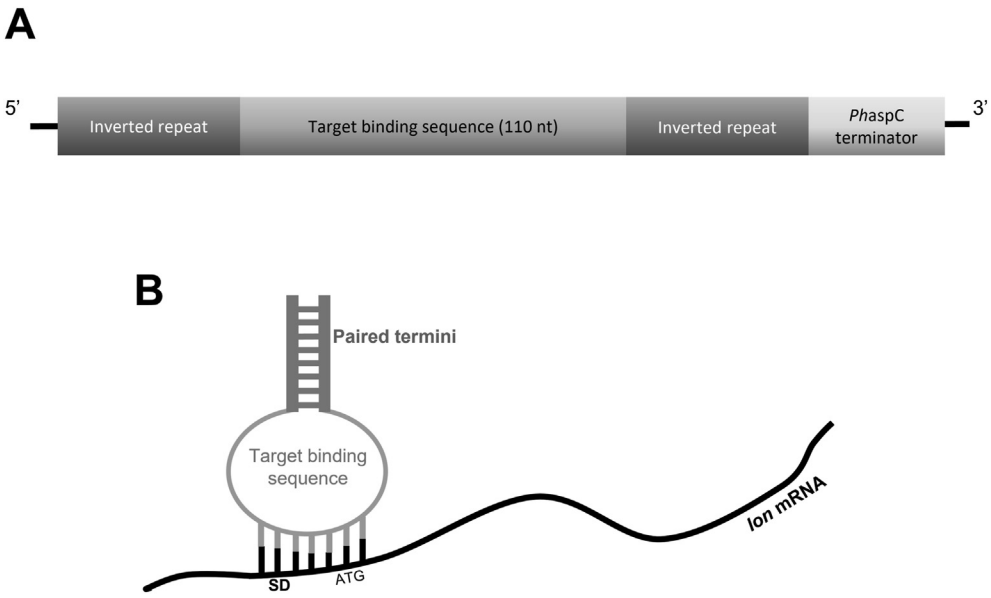

Fig. 2. Schematic representation of PTasRNA. (A) The antisense sequence, complementary to the TIR of *lon* mRNA, was included between two inverted repeats. **(B)** The inverted repeats at the ends of the sequence form a hairpin structure containing the antisense sequence of the target gene to be silenced into the loop. SD, Shine-Dalgarno sequence, ATG, translation start site.

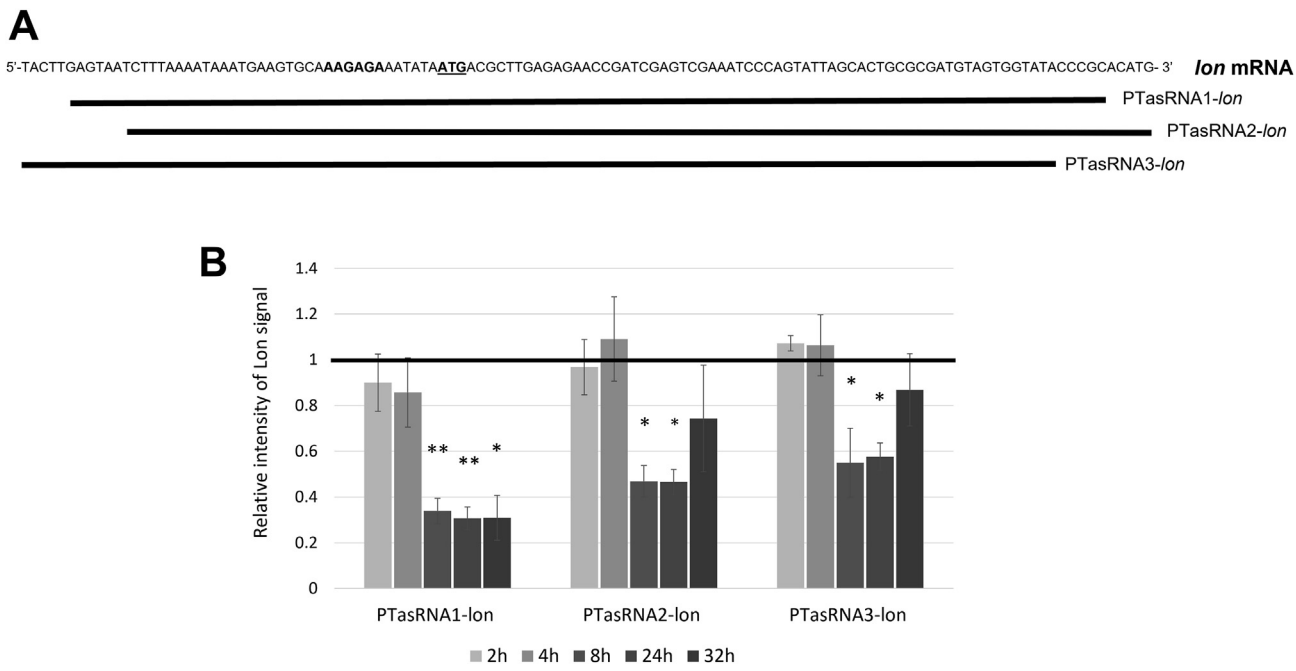

Fig. 3. Evaluation of *Phlon* gene silencing mediated by PTasRNA-*lon*. **(A)** Schematic illustration of the antisense fragment positions of PTasRNA-*lon* variants. The fragment positions are shown as bars covering the *Phlon* mRNA, whose predicted SD and translation start site are reported in bold and underlined bold, respectively. PTasRNA1-*lon* and PTasRNA2-*lon* were designed with a shift of 5 and 10 nt of the PTasRNA3-*lon* antisense sequence towards *Phlon* mRNA 3′-end, respectively. **(B)** Evaluation of relative cellular concentration of Lon protease in cells expressing the three variants of PTasRNA-*lon* in comparison to control cells, carrying the empty vector. Western blotting was performed on total protein extracts of cells recovered 2, 4, 8, 24 and 32 h after the induction of asRNA-*lon* transcription using anti-EcLon antibodies. The black bar represents the baseline amount of Lon detected in the control samples. The measurements are reported as the mean of two independent experiments whose standard deviation is indicated by the error bars. The data were considered significant when $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) according to t-Student test.

harboring the empty vector - used as positive controls – showed a clear peroxide-resistant phenotype, characterized by an almost negligible killing zone. In the same experimental conditions the *Ph*TAC125-30 knockout mutant [25] was sensitive to the oxidative stress, and its phenotype in the disk-diffusion assay was used to evaluate if the *PhhbO* conditional gene silencing was achieved (Fig. 5 and Table 3). Interestingly, the PTasRNA-*hbO* expression was coupled to an H_2O_2 growth inhibition comparable to the one observed when the *Ph*TAC125 knockout mutant was tested (see Table 3), suggesting that the *PSHA_RS00150* gene silencing reached a level so high to be considered as total repression.

4. Discussion

The availability of several techniques for genome-wide genetic manipulations in common bacterial hosts paved the way for targeted strain engineering in the framework of both fundamental and applied studies. However, the application of such methods in environmental strains still represents a challenging task. *P. haloplanktis* TAC125 (*Ph*TAC125) is a model for environmental studies because of its notable adaptation to low temperatures lifestyle [19–21]. Despite this psychrophilic bacterium has been successfully used as a non-conventional platform for the

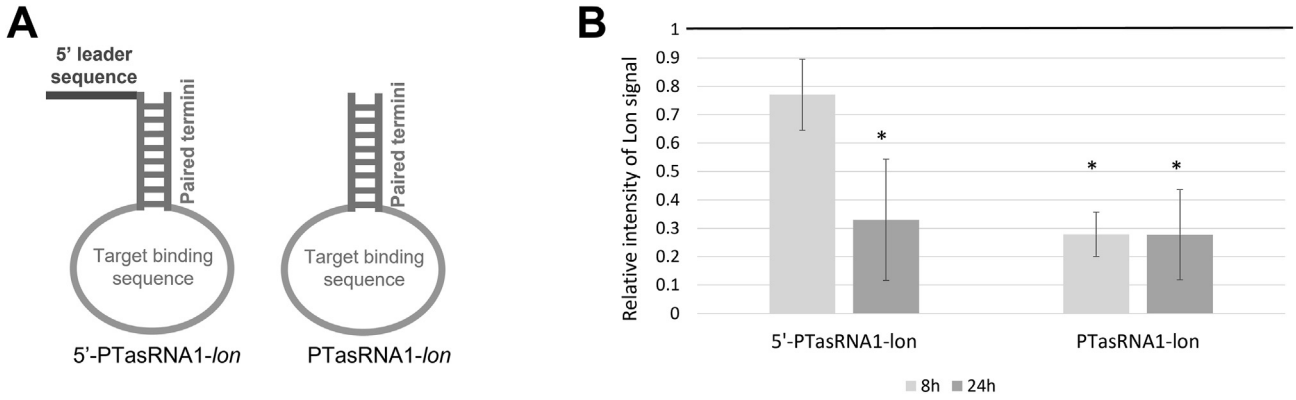

Fig. 4. Evaluation of the effect of a 5′ leader sequence upstream of the hairpin structure ofPTasRNA1-*lon* **(A)** Schematic representation of 5′-PTasRNA1-*lon* construct. The 5′ leader sequence derived from *PhlacZ* was placed upstream ofthe inverted repeat involved in the formation of the hairpin structure, whose loop holds the antisense sequence. **(B)** Evaluation of the relative cellular concentration of Lon protease in cells expressing either 5′-PTasRNA1-*lon* orPTasRNA1-*lon* in comparison to control cells, carrying the empty vector. Western blotting was performed on total protein extracts of cells recovered 8 and 24 h after the induction of asRNA-*lon* transcription using anti-EcLon antibodies. The black bar represents the baseline amount of Lon detected in the control samples. The measurements are reported as the mean of two independent experiments whose standard deviation is indicated by the error bars. The data were considered significant when $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) according to t-Student test.

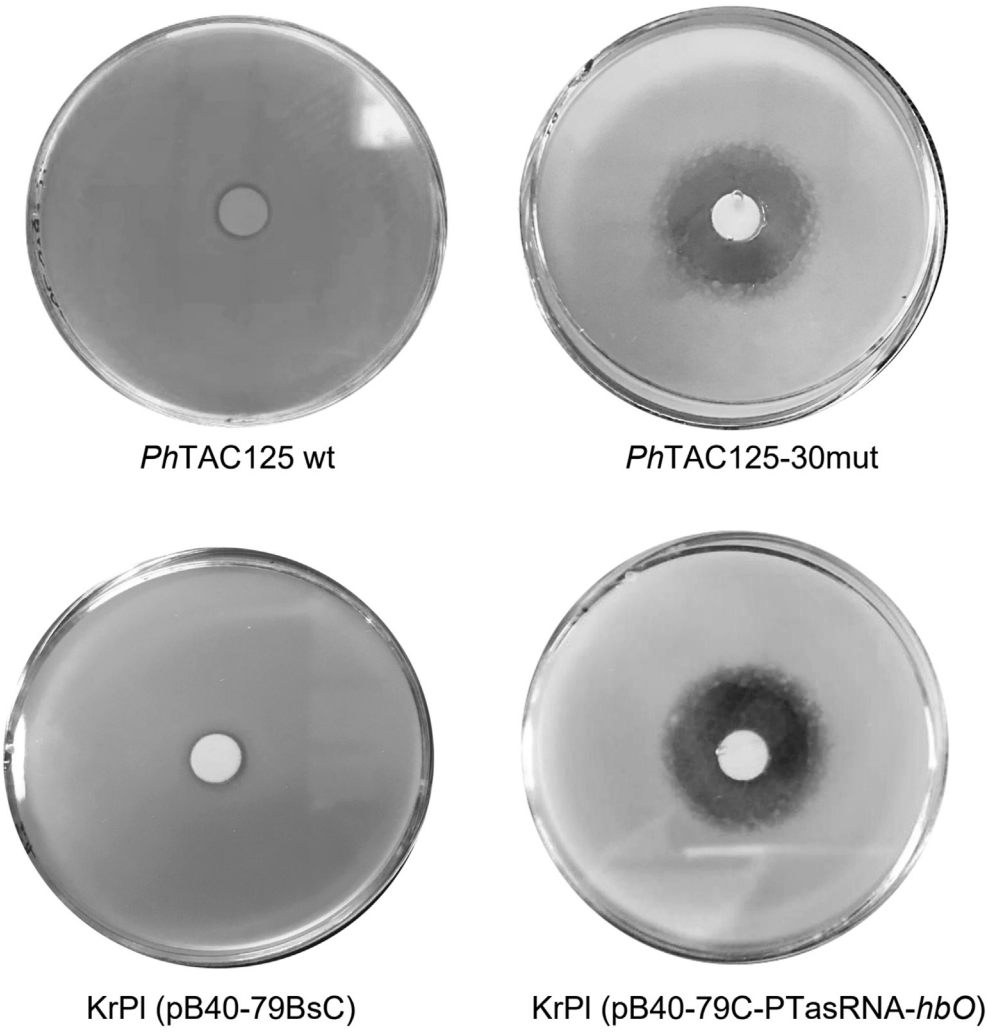

Fig. 5. Evaluation of PTasRNA-*hbO* expression on hydrogen peroxide sensitivity of recombinant *Ph*TAC125 cells. Disk diffusion assay in the presence of H_2O_2 (265 mM) of *Ph*TAC125, *Ph*TAC125-30mut, KrPL pB40-79BsC and KrPL pB40-79C-PTasRNA-*hbO* grown for 24 h at 15 °C. The peroxide sensitivity is directly proportional to the diameter of the killing zone (the dark halo) surrounding the Watmann paper disk.

recombinant production of difficult-to-produce proteins [32–36], the only consolidated genome mutagenesis technology is based on a two-steps integration-segregation procedure relying on homology recombination [22]. Nevertheless, the frequency of the second recombination event is often very low, and the "clean" gene deletion is a rare event. Furthermore, this disruption method is not suitable for the study of essential genes.

The present study intended to develop effective methods for conditional gene silencing in *Ph*TAC125 using synthetic antisense RNAs. Based on their mode of action, two kinds of asRNAs were evaluated: Hfq-dependent asRNA and Paired Termini asRNA. These synthetic molecules were challenged for their ability to suppress the translation of the Lon protease encoding mRNA.

Although no endogenous regulatory asRNAs of *Ph*TAC125 have been identified so far, the Hfq-dependent asRNA strategy was pursued relying on the observed high identity degree (85%) between the *Ph*TAC125 and *E. coli* Hfq amino acid sequences (Fig. S4). However, our results demonstrated that MicC scaffold-based asRNA was able to interfere with the *lon* gene expression only after 2 h post-induction, while no *lon* silencing was evident at later time points. As the cellular Hfq concentration in bacterial cells is generally limiting, it is important that Hfq leaves the mRNA-asRNA complex upon formation, to exert its RNA chaperone function on other molecules. Most bacterial Hfqs, including the *E. coli* one, possess an intrinsically disordered region (IDR) in the C-terminal domain (CTD), which is needed to displace base-paired RNAs, so as to allow Hfq recycling [40]. *Ph*TAC125 Hfq is characterized by a shorter CTD, in which the predicted IDR domain is almost totally deleted (Fig. S5). Our hypothesis is that the psychrophilic Hfq binds MicC asRNA and stabilize its interaction with *lon* mRNA, but lacking the disordered region in its CTD, it could be unable to leave the complex, resulting (after only 2 h post-induction) in the reduction of the cellular concentration of the free chaperone.

We benefited from the structural features of PTasRNAs to obtain successful and efficient gene silencing in *Ph*TAC125. The different efficiency of *lon* down-regulation achieved by the three PTasRNA-*lon* variants (differing in 5′ and 3′ interaction boundaries) confirmed that the predicted binding energy is a critical parameter to establish an efficient gene control system also at low temperatures. Our finding is in agreement with what was described by Nakashima and coworkers [11]. Indeed, they reported that slightly different targeting sequences could influence the efficiency of down-regulation of *lacZ* mRNA but a general design rule was not assessed. Furthermore, a 5' leader sequence upstream of PTasRNA-*lon* did not improve its repression capability. It can be concluded that the hairpin setup of PTasRNA is

sufficiently stable to induce the desired effect also in the psychrophilic bacterium.

However, a complete silencing of *lon* was never observed in our experimental conditions. To highlight the potential bottlenecks, if any, limiting the gene silencing outcomes, we focused our attention on the relative abundance of the target mRNA and PTasRNA. Indeed, a molar excess of asRNA is essential for efficient gene silencing, to out-compete ribosomes for binding with the target transcript [41]. Since a transcriptomic analysis of *Ph*TAC125 cells grown at 15 °C placed the *lon* gene among the 140 most transcribed ones (unpublished data from this laboratory), we hypothesized that our gene expression system was unable to establish a sufficient PTasRNA-*lon* molar excess. However, total silencing could be obtained targeting a less expressed gene. A good candidate for this new trial was the *PhhbO* gene, which is transcribed about 40 times less than *lon* (unpublished data from this laboratory). Interestingly, the psychrophilic cells expressing PTasRNA-*hbO* display the same peroxide-sensitive phenotype presented by the knockout mutant [25], thus supporting a compelling reduction of trHbO protein concentration likely due to the total repression of *hbO* mRNA translation.

In this work, we defined the guidelines for designing a conditional gene control system based on asRNA in *P. haloplanktis* TAC125. For the first time we demonstrated that the down-regulation of two genes (selected as transcribed at quite different levels) is feasible also in a wild type Antarctic marine bacterium, one of the widely acknowledged models for studying bacterial adaptation to freezing lifestyle.

Ethical statements

There are no ethical issues relevant to this work.

Declaration of competing interest

There are no conflicts of interest with authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2022.103939.

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