

Article

Targeted Regulation of Protein Expression in *Vibrio parahaemolyticus*

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Simple Summary

Cellular phenotypes arise from quantitative and qualitative changes in proteins. Here, we developed an arabinose-regulated effector protein expression system in *Vibrio parahaemolyticus* and showed that VP1680 expression enhances cytotoxicity to mammalian cells in a dose-dependent manner. We also established a targeted protein degradation system using VP0917 (ClpP), VP0918 (ClpX), and VP1014 (ClpA) to degrade ssrA-tagged proteins, achieving over 50% degradation within 20 min. As a byproduct, we obtained an enhanced green fluorescent protein (EGFP) variant with strong fluorescence in *V. parahaemolyticus*. This variant serves as a useful tool for visualizing intracellular proteins. These tools enable precise manipulation of intracellular protein levels, offering new ways to explore and redirect bacterial physiology.

Abstract

V. parahaemolyticus has several virulence factors, including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), and two separate type III secretion systems (T3SSs), T3SS1 and T3SS2. T3SS1 is responsible for cytotoxicity, primarily through the activity of its effector VP1680. To gain a detailed understanding of the relationship between the amount of effector, its expression timing, and cytotoxicity, a system is required to regulate protein expression levels and timing. In the present study, we developed an effector protein expression system controlled by an arabinose-dependent transcription factor and found that cytotoxicity toward mammalian cells increased in a VP1680-dependent manner. To ensure specific protein degradation, we also established a targeted protein degradation system, including VP0917 (ClpP) and VP0918 (ClpX)-, or VP0917 and VP1014 (ClpA)-mediated degradation of ssrA-tagged proteins (proteins bearing the C-terminal degradation tag encoded by tmRNA). By combining these systems, more than 50% of the targeted protein could be degraded within 20 min. As a byproduct of creating the systems, we obtained an enhanced green fluorescent protein variant that emits strong fluorescence in *V. parahaemolyticus*. The protein degradation system developed in this study has demonstrated the potential to control intracellular protein levels to a certain extent. Moreover, experimentally controlling intracellular protein levels will allow for a more detailed examination of the relationship between protein quantity and cellular phenotype, potentially overcoming the limitations of the “all-or-nothing” model.



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1. Introduction

To date, various synthetic biology tools have been developed and used to control protein levels in cells [1]. At present, the following methods can be regulated using synthetic biology tools: (1) regulation of mRNA transcription levels, (2) regulation of translation efficiency by redesigning the ribosome binding site, and (3) regulation of the rate of protein degradation [1–4]. The transcriptional regulatory system can control the amount and timing of protein expression in cells by flexibly modulating mRNA levels [1]. By combining transcriptional regulation with a protein degradation system, protein expression can be achieved at any desired time point, enabling a more detailed evaluation of protein function.

Vibrio parahaemolyticus is a halophilic, Gram-negative bacterium, and it mainly lives in seawater. Consumption of seafood contaminated with *V. parahaemolyticus* can cause food poisoning characterized by diarrhea, abdominal pain, vomiting, and fever [5]. *V. parahaemolyticus* has several virulence factors, including thermostable direct hemolysin (TDH); TDH-related hemolysin (TRH); and two separate type III secretion systems (T3SSs), T3SS1 and T3SS2 [6,7]. T3SSs are protein export systems that secrete and translocate effectors into the cytoplasm of host cells. Translocated effectors modify host cell function, enabling pathogens to exert pathogenicity [8,9]. T3SS1 is responsible for cytotoxicity, whereas T3SS2 is associated with enterotoxicity in mammals [10–12]. Cytotoxicity following *V. parahaemolyticus* infection is primarily due to the activity of the T3SS1 effector VP1680 (also known as VepA and VopQ), which is both necessary and sufficient to induce an inflammatory response and cell death [13–15]. Previously, we found that VP1680 expression levels contribute to cytotoxicity independently of growth speed [16]. In addition, T3SS1 was regulated in a cell density-dependent manner [17]. However, to gain a detailed understanding of the relationship among cell density, the amount of effector, its expression timing, and pathogenicity, a system is required to regulate protein expression levels and timing.

Systems utilizing transcriptional regulation to control intracellular protein expression levels have been reported in *Escherichia coli* [18]. These systems combine specific transcription factors with promoter regions and control transcriptional activity by modulating transcription factor binding to promoters with small-molecule compounds. For example, the pBAD/AraC system is a transcriptional regulatory system that combines the arabinose operon promoter BAD with the transcription factor AraC. In the absence of arabinose, AraC binds to the araI and araO2 sites near the promoter, forming a DNA loop. It inactivates the BAD promoter, thereby suppressing expression of the araBAD operon. In the presence of arabinose, AraC binds to arabinose, breaking the DNA loop, activating the BAD promoter, and inducing expression of the araBAD operon [19,20]. Although transcription regulation systems have been reported in many Gram-negative bacteria, such as *E. coli*, *Salmonella*, and *Xanthomonas* [21–23], no such system has been reported in *V. parahaemolyticus*.

The existence of ATP-dependent proteases that regulate substrate-specific proteolytic reactions, such as ClpXP and ClpAP in *E. coli*, has been clarified [24]. These ATP-dependent proteases unfold substrate proteins using ATP and transfer them to the protease domain for degradation. For example, ClpXP, one of the ATP-dependent proteases, is composed of the ATPase subunit ClpX and the peptidase subunit ClpP [25]. ClpX binds to the ssrA-tag (AANDENYALAA), which is composed of 11 amino acids and is attached to the C-terminus of the substrate protein [25], and unfolds the three-dimensional structure of the substrate protein. Furthermore, this denatured polypeptide is transferred to ClpP for degradation. The ClpXP-mediated protein degradation reaction, triggered by recognition of the ssrA-

tag, plays a vital role in the trans-translation response in bacteria. Trans-translation is a series of reactions that rescue ribosomes that have stopped functioning due to incomplete translation. When an mRNA loses its stop codon due to mutation, the ribosome reaches the 3' end of the mRNA but is unable to terminate translation, becoming stuck on the mRNA. Trans-translation aims to remove the mRNA from the stuck ribosome [26]. The central player in this reaction is tmRNA, which acts as both tRNA and mRNA. tmRNA enters the A site of the stalled ribosome and translocates to the neighboring P site, removing the mRNA from the ribosome. Next, the *ssrA*-tag sequence present in tmRNA is translated, and the *ssrA*-tag is added to the C-terminus of the protein whose translation has stopped partway through, and translation is terminated. The tagged protein is released from the ribosome and degraded by an ATP-dependent protease (mainly ClpXP). The released ribosome is reused for the following translation. Experimentally, *trpAt*-terminator tag (gcagccgccuaagagcgggcuuuuuu) caused ribosomal stalling and cleaved by RNase, tagged *ssrA* peptide by *smpB* and tmRNA, *ssrA*-tag is recognized and proteolyzed by ClpAP, ClpXP, LonP, and HsvI [4,27]. However, no methods for regulating intracellular protein levels via targeted protein degradation have been reported in *V. parahaemolyticus*.

If we can establish transcriptional regulatory and targeted protein degradation systems in *V. parahaemolyticus*, we can regulate effector protein expression and investigate the relationship between the number of effector proteins and virulence. In other words, it will be possible to investigate whether the relationship between the number of effector proteins and virulence is linear, and whether the more effector proteins there are, the stronger the virulence, or whether there is a threshold value below which virulence is not exhibited. In addition, it will be possible to investigate the relationship between the timing of effector protein expression and virulence. The aim of this research is (1) to establish a transcriptional regulatory system to control the expression level of effector proteins; (2) to investigate effect of different number of effector on virulence of *V. parahaemolyticus* using the established system; (3) to establish a targeted protein degradation system, including identification of the ATP-dependent protease involved in degradation reactions mediated by the *ssrA*-tag, for *V. parahaemolyticus*; and (4) to create a system for controlling timing of protein expression.

2. Materials and Methods

2.1. *V. parahaemolyticus* Strains and Culture Conditions

V. parahaemolyticus (RIMD2210633) and its mutant strains (Supplemental Table S1) used in this study were grown in 3% NaCl LB medium (1% Tryptone (Nacalai Tesque, Kyoto, Japan), 0.5% Yeast Extract (Nacalai Tesque)) with antibiotics added as necessary, and shaken at 37 °C. Plasmids pHRP309 and pSA19 used in this study are listed in Supplemental Table S1. To express the target gene, we used the promoter of the thermostable hemolysin A (*tdhA*) gene, which has already been reported to have high transcriptional activity in *V. parahaemolyticus*. The mutant strains were constructed by homologous recombination as previously described using specific primers (Supplemental Table S2) [16]. Briefly, ~500-bp fragments immediately upstream and downstream of the gene of interest were amplified by PCR. These two fragments were joined by PCR and cloned into the pYAK vector. The resulting plasmid was transformed into *V. parahaemolyticus* by conjugation. The deletion mutant was screened by antibiotics and sucrose.

2.2. Electroporation for Plasmid Transfection

Overnight cultured *V. parahaemolyticus* was diluted 200 times with 0.5% NaCl LB medium and cultured at 37 °C for 2.5 h with shaking (170 rpm); 2 mL of the bacterial solution was centrifuged (12,000 rpm, 2 min). After removing the supernatant, it was

washed with electroporation (EP) buffer (272 mM sucrose, 7 mM Na-phosphate buffer (pH 7.4), 1 mM MgCl₂). After washing 3 times, the bacteria were resuspended in 100 µL of EP buffer; 100 ng of plasmid was added and electroporation was performed using the Gene Pulser Xcell (BIO-RAD Laboratories, Hercules, CA, USA). After electroporation, bacterial solution was added to 1 mL of 1% NaCl LB medium and shaken at 37 °C for 1 h (170 rpm). Bacterial solution was plated on a 3% NaCl LB plate containing gentamycin (10 µg/mL) for pHRP309 or chloramphenicol (10 µg/mL) for pSA19 and incubated overnight at 37 °C.

2.3. Fluorescence Intensity Measurement

Bacteria carrying plasmids encoding enhanced green fluorescent protein (EGFP) and its variants were cultured at 37 °C for 24 h in 2 mL of 3% NaCl LB medium with shaking (170 rpm); 1 mL of this bacterial solution was centrifuged (12,000 rpm, 2 min), and the supernatant was removed; the pellet was resuspended in 190 µL of phosphate-buffered saline (PBS). This was transferred to a 96-well plate, and the relative fluorescence intensity (Relative Fluorescence Units: RFU) was measured at 488 nm excitation and 507 nm emission (or an appropriate excitation/emission wavelength) using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Bremen, Germany).

2.4. Western Blotting

One milliliter of *V. parahaemolyticus* cultured after arabinose stimulation for the indicated time at 37 °C was centrifuged (4 °C, 12,000 rpm, 3 min), and the supernatant was removed. Cellular protein was extracted, and Western blotting was performed using a general method as previously reported, with anti-GFP antibody (WAKO, Osaka, Japan, mF75) or anti-FtsA antibody (Sigma-Aldrich, St. Louis, MO, USA) [16]. Briefly, the cultures were centrifuged at 12,000× *g* for 3 min, and the cell pellets were resuspended in RIPA buffer. After electrophoresis in a polyacrylamide gel, proteins were transferred to a PVDF membrane (Immobilon-P, Merck Millipore, MA, USA). The membrane was blocked using 1% skim milk and probed with a specific antibody.

2.5. RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR

RNA extraction, cDNA synthesis, and quantitative RT-PCR were performed using a general method as previously reported [16]. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by using a PrimeScript reverse-transcription (RT) reagent kit (TaKaRa Bio Inc., Shiga, Japan) and quantitative RT-PCR analysis was performed.

2.6. Infection of HeLa Cells and Measurement of Cytotoxicity

Infection of HeLa cells was performed as previously reported [16]. Briefly, *V. parahaemolyticus* strains were harvested from overnight cultures and pelleted by centrifugation at 6000× *g* at room temperature. The pellets were washed 3 times using PBS, adjusted to an OD₆₀₀ of 1.0, and inoculated into the HeLa cells at a multiplicity of infection of 1. Supernatants of culture medium were collected at 6 h postinfection, and lactate dehydrogenase (LDH) activity was measured with a cytotoxicity detection kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.7. Statistics

All values are expressed as the mean ± SE. The differences among more than two groups was tested using ANOVA or the Kruskal–Wallis test. When a significant difference was found by ANOVA or the Kruskal–Wallis test, post analyses were performed using the Tukey–Kramer protected least significant difference test. Differences were considered significant at *p* < 0.05. Statistical analyses were performed using Excel TOKEI 2010.

3. Results

3.1. Development of an Efficient EGFP Variant for *V. parahaemolyticus*

Before developing a protein expression regulation system, we tried to improve EGFP fluorescence. We modified some amino acids in EGFP, as observed in the brighter fluorescence protein Venus [28]. We generated six EGFP variants and checked their fluorescence under a constitutively active *tdhA* promoter. Among six variants, EGFP-variant2 (EGFP-V2), which contains the M153T, V163A, and S175G mutations, shows 30-fold higher fluorescence than EGFP (Figure 1). In subsequent studies, we used EGFP-V2.



Figure 1. Development of an efficient EGFP variant for *V. parahaemolyticus*. Relative fluorescence of EGFP and EGFP variant in *V. parahaemolyticus*. The indicated amino acid was replaced in each EGFP variant. Venus has 10 amino acids mutation (F46L, T65G, V68L, S72A, R80Q, M153T, V163A, S175G, T203Y, H231L) from EGFP. Results were shown as mean $\pm$ SE. Different letters indicate $p < 0.05$ by ANOVA.

3.2. Development and Characterization of an Inducible Expression System in *V. parahaemolyticus*

To develop a protein expression regulation system, we next developed arabinose-inducible expression plasmids (Figure 2A). We chose low-copy plasmid pHRP309 and high-copy plasmid pSA19 and constructed these plasmids to contain the transcription factor AraC, which can be activated by arabinose, subsequently binds to the BAD promoter, and promotes expression of the downstream target gene. The expression of the downstream target gene was increased in arabinose stimulation and suppressed by glucose stimulation in both plasmids expressing *V. parahaemolyticus* (Figure 2B). We found leaky expression of the EGFP-V2 in the pSA19 plasmid but not in the pHRP309 plasmid (Figure 2B). We chose pHRP309 to investigate the detailed expression pattern. The induced activity of this system with 0.05% arabinose was 69% of that of the *tdhA* promoter, and no induction was observed without arabinose in the pHRP309 plasmid (Figure 2C). This promoter was induced in an arabinose dose-dependent manner in both mRNA expression and relative fluorescence levels (Figure 2D,E). On the time scale, induction of mRNA was observed at 15 and 30 min after 0.05% arabinose stimulation, and fluorescence increased time-dependently until 240 min after stimulation (Figure 2F,G). This plasmid system cannot repeatedly induce target mRNA expression (Figure 2H). These results clearly showed the character of this system, which can express the target protein at the indicated time.

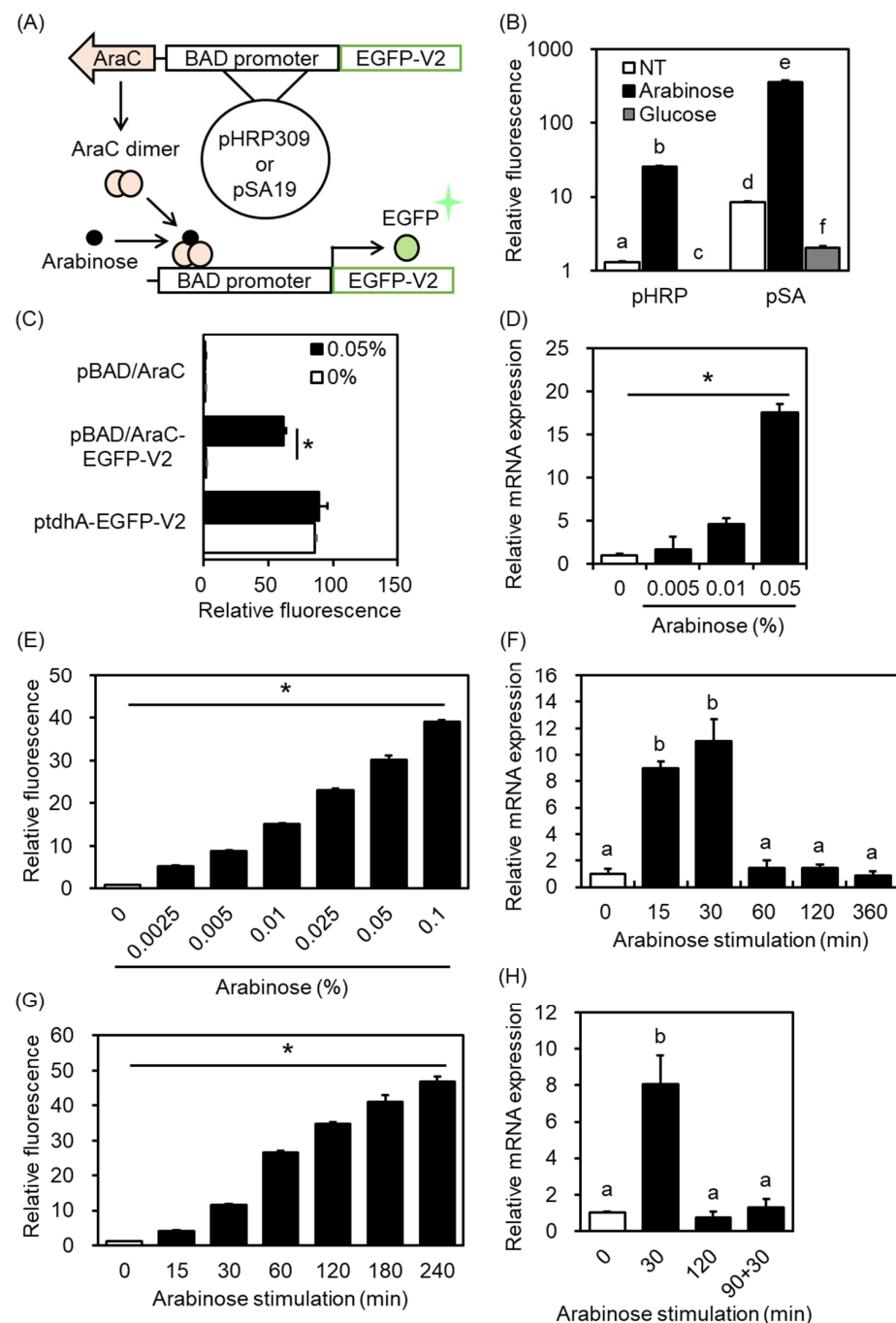


Figure 2. Development and characterization of an inducible expression system in *V. parahaemolyticus*. (A): Design of plasmid-based inducible expression system. Plasmids (pSA19 and pHRP309) carrying EGFP-variant under BAD promoter, which is induced by AraC and arabinose in a concentration-dependent manner. (B): Relative fluorescence of bacteria expressing pHRP309-pBAD/AraC-EGFP-V2 and pSA19-pBAD/AraC-EGFP-V2 cultured 24 h with or without 0.05% arabinose or 0.2% glucose. (C): Relative fluorescence of bacteria expressing pHRP309-pBAD/AraC, pHRP309-pBAD/AraC-EGFP-V2, and ptdhA-EGFP-V2 cultured 24 h with or without 0.05% arabinose. (D,E): Relative mRNA expression (D) and fluorescence (E) of bacteria expressing pHRP309-pBAD/AraC-EGFP-V2 cultured 30 min (for mRNA), 24 h (for fluorescence) with the indicated dose of arabinose. (F–H): Relative mRNA expression (F,H) and fluorescence (G) of bacteria expressing pHRP309-pBAD/AraC-EGFP-V2 cultured for the indicated time with 0.05% arabinose. 90 + 30 sample stimulated 0.05% arabinose at time 0 and 90 min and measured the sample at 120 min after the first stimulation. Results were shown as mean $\pm$ SE. *: $p < 0.05$ between groups by t -test in (C). *: $p < 0.05$ in linear regression analysis in (D,E,G). Different letters indicate $p < 0.05$ by ANOVA.

3.3. Characterization of Trans-Translation System-Mediated Protein Degradation in *V. parahaemolyticus*

Next, we consider the protein degradation system to control the shutdown of protein expression. To achieve this, we focused on a trans-translation system and used trpAt-terminator tag, ssrA-tag, and ssrHis tag, which was replaced with the final six amino acids of ssrA-tag to histidine (TSAANDHHHHHH). Compared to the fluorescence of EGFP-V2-ssrHis, EGFP-V2-ssrA did not show fluorescence, suggesting *V. parahaemolyticus* has a proteolysis system for ssrA-tagged protein (Figure 3A). To search for proteases targeted for EGFP-V2-ssrA in *V. parahaemolyticus*, we constructed an independent deletion mutant suspected of ssrA-tagged protein breakdown. Among six mutants, the VP0917 (ClpP) and VP0918 (ClpX) deletion strains exhibited fluorescence from EGFP-V2-ssrA (Figure 3B). The VP0917 deletion strain has higher fluorescence than the VP0918 deletion strain, suggesting additional genes co-work with these genes. Next, we developed double- and triple-deletion strains and found that deletion of both VP0918 and VP1014 (ClpA) has an additive effect, whereas deletion of other genes has no additive effect on the VP0918 deletion strain (Figure 3C). We also confirm that deletion of both VP0918 and VP1014 results in fluorescence from EGFP-V2-ssrA that is similar to that of EGFP-V2-ssrHis (Figure 3D). In contrast to EGFP-V2-ssrA, the EGFP-V2-trpAt-expressing bacteria exhibit fluorescence similar to EGFP-V2-ssrHis. We confirmed the reliability of trpAt-tag in the tmRNA deletion strain and found EGFP-V2-trpAt has higher fluorescence in the tmRNA deletion strain (Figure 3E). These results suggest that VP0918 and VP1014 recognized and VP0917 proteolyzed the ssrA-tagged protein, and some of the EGFP-V2-trpAt escaped the trans-translation system in *V. parahaemolyticus* (Figure 3F). We decided to use the ssrA-tag and VP0917-VP0918 to control protein degradation.

3.4. Development and Characterization of Inducible Protein Expression and Degradation System in *V. parahaemolyticus*

Finally, we investigated the cytotoxicity of *V. parahaemolyticus* using an arabinose-dependent VP1680 expression system in a VP1680 deletion mutant (Figure 4A). In this strain, cytotoxicity to mammalian cells increased in a concentration-dependent manner with arabinose (Figure 4B). Next, we investigated the time required for protein breakdown in our system (Figure 4C). We use two plasmids: one expresses the EGFP-V2-ssrA protein under the *tdhA* promoter, and the other expresses VP0917-VP0918 under the pBAD promoter, in the VP0917-VP0918 deletion strain. In this strain, EGFP-V2-ssrA fluorescence can be observed in the absence of arabinose, but it was turned off by arabinose treatment (Figure 4D). EGFP-V2-ssrA protein was reduced in a time-dependent manner with a half-life of around 20 min after arabinose treatment (Figure 4E). These examples will help to control the number- and timing-dependent cellular functions in *V. parahaemolyticus*.

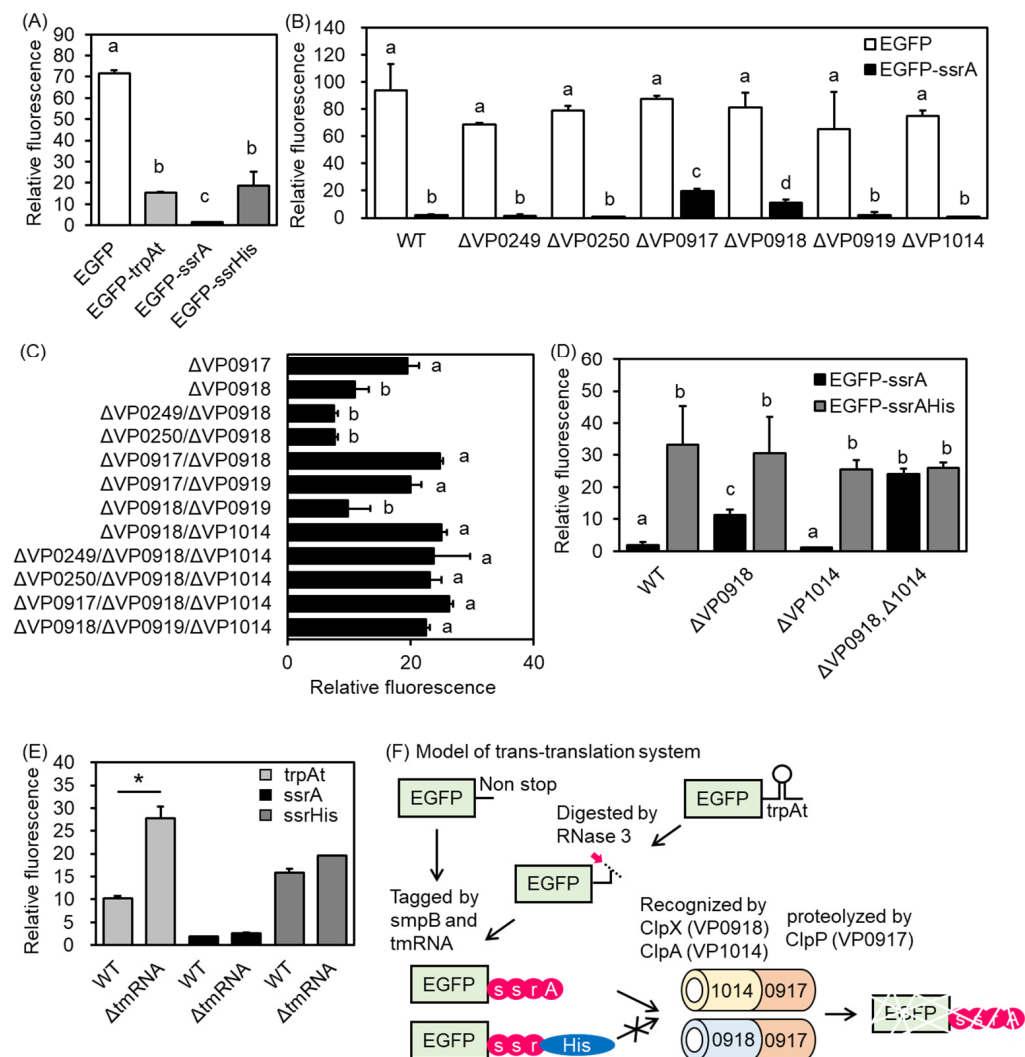


Figure 3. Characterization of the trans-translation system-mediated protein degradation in *V. parahaemolyticus*. **(A)**: Relative fluorescence of bacteria strains pHRP309-EGFP-V2 with or without a different sequence or tags. **(B)** Relative fluorescence of each bacterial strain, pHRP309-EGFP-V2 with or without ssrA-tag. **(C)**: Relative fluorescence of each deletion mutant, pHRP309-EGFP-V2 with ssrA-tag. **(D)**: Relative fluorescence of each bacterial strain, pHRP309-EGFP-V2 with ssrA or ssrHis tag. **(E)**: Relative fluorescence of each bacterial strain, pHRP309-EGFP-V2 with a different sequence or tags. **(F)** Model of the trans-translation system in *V. parahaemolyticus*. Results were shown as mean $\pm$ SE. *: $p < 0.05$ between groups by *t*-test in (E). Different letters indicate $p < 0.05$ by ANOVA.

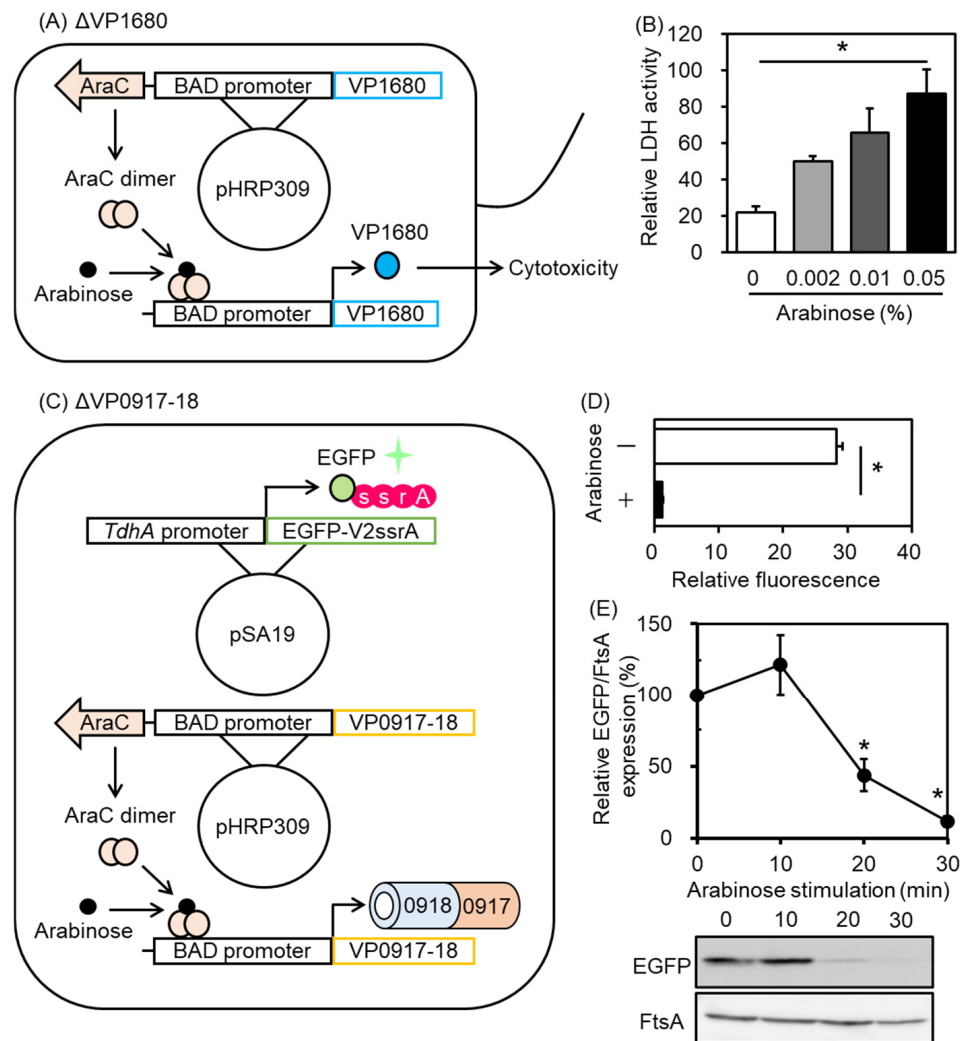


Figure 4. Development and characterization of an inducible protein expression and degradation system in *V. parahaemolyticus*. **(A)**: Design of plasmid-based inducible VP1680 expression system in VP1680 deletion mutant. **(B)**: Relative LDH activity in the culture medium of HeLa cells infected with bacteria shown in **(A)** with the indicated arabinose concentration for 6 h. **(C)**: Design of plasmid-based inducible VP0917-VP0918 expression system with constitutively expressed EGFP-V2-ssrA in VP0917-VP0918 deletion mutant. **(D)**: Relative fluorescence of bacteria shown in **(C)** with or without arabinose supplementation. **(E)**: Time course of changes in EGFP-V2-ssrA expression in bacteria shown in **(C)** after 0.05% arabinose stimulation. Results were shown as mean $\pm$ SE. *: $p < 0.05$ in linear regression analysis in **(B)**. *: $p < 0.05$ between groups by *t*-test in **(D)**. *: $p < 0.05$ in ANOVA with Dunnett post hoc test compared with time 0 in **(E)**.

4. Discussion

Quantitative and qualitative alterations in protein expression drive changes in cellular phenotype. In the present study, we developed an effector protein expression system regulated by arabinose and found that cytotoxicity against mammalian cells increased in a VP1680 expression-dependent manner. We also established a targeted protein degradation system, including VP0917 and VP0918- or VP0918 and VP1014-mediated degradation of *ssrA*-tagged proteins. By combining these systems, more than 50% of the targeted protein could be degraded within 20 min. As a byproduct of creating the systems, we obtained an EGFP variant that emits strong fluorescence in *V. parahaemolyticus*. The tagged protein specific degradation systems for controlling protein expression in cells could be useful for investigating an alternative physiological role of this bacterium.

Our data showed that EGFP mRNA expression peaked at approximately 30 min and subsequently decreased in the arabinose utilization system. In contrast to mRNA expression, the RFU of EGFP-V2 or cytotoxicity to HeLa cells increased over time. Based on these findings, the temporal and quantitative changes in mRNA expression achieved by the transcriptional regulation system using the pBAD/AraC system in *V. parahaemolyticus* established in this study are considered controllable, as in other bacteria [20,23].

Using an arabinose-regulated transcription system, we investigated whether the expression level of the cytotoxicity-related gene VP1680 could be controlled. The results showed that cell death was induced in an arabinose concentration-dependent manner. That is, under the conditions tested, the number of effector proteins and cytotoxicity appeared to have a linear relationship, suggesting that increased effector protein levels correlate with stronger cytotoxicity. This system might be used to study how many effector proteins are needed to induce cytotoxicity. In this experiment, infection was performed for 6 h. Since experiments tracking infection over time were not conducted, future studies should examine cell death induction at different stimulation times to determine the optimal timing for effector protein expression to induce cell death.

Previous studies have demonstrated that in *E. coli*, ATP-dependent proteases such as ClpXP, ClpAP, and Lon are involved in the degradation of proteins tagged with the *ssrA* [29,30]. In the present series of deletion mutant experiments, we found that ClpXP (VP0918 and VP0917) and ClpAP (VP1014 and VP0917) may be involved in the degradation of *ssrA*-tagged proteins in *V. parahaemolyticus*. Unlike in *E. coli*, we confirmed that there was no association between the degradation of *ssrA*-tagged proteins by Lon protease (VP0919) in *V. parahaemolyticus* [4]. The fact that dual deletion of VP0918 and VP1014 more strongly inhibited degradation than VP0918 deletion alone, and that VP1014 deletion alone did not inhibit degradation, suggests that VP0917-VP0918 primarily drives the degradation of *ssrA*-tagged proteins, with VP0917 and VP1014 playing a supplementary role in *V. parahaemolyticus*. By using strains with different degradation efficiencies, obtained by deleting these proteases, we established a protein degradation system capable of stepwise control.

In previous research, when investigating the function of a specific protein, researchers created a deletion mutant of the gene of interest and analyzed the phenotype of those cells [15,16]. However, it is rare for a particular protein to be completely absent from a cell, and in cells, protein levels are cleverly controlled by regulating expression and degradation to control its function. Therefore, to investigate the function of a specific protein under physiological conditions, it is essential to maintain the protein's concentration. Our system may help to regulate the amount of the protein of interest in some cases. We expect that establishing systems for regulating protein levels will contribute to understanding the pathogenicity and its mechanisms of various bacteria, including *V. parahaemolyticus*. Additionally, one advantage of our present study is that we have identified a 30-fold brighter EGFP variant in *V. parahaemolyticus*. Rekas A et al. reported that M153T, V163A, and S175G improve the rate of maturation [28]. These mutations could facilitate the protein folding process in *V. parahaemolyticus*. To use this variant, we can visualize the protein of interest at 30-fold magnification. This variant could achieve higher protein resolution within a cell, enabling single-protein or single-cell analysis to solve the relationship between the number of pathogenic factors and pathogenicity in *V. parahaemolyticus*.

Our system has some limitations. First, because our proteolytic system uses a VP0917-18 deletion strain, it is difficult to consider this protease in a physiological context. Previously, several *ssrA*-tag mutants with different degradation rates have been created in *E. coli* [31]. In *V. parahaemolyticus*, this issue could potentially be resolved by creating tag mutants with various degradation rates, in addition to the control tag developed in this study. Second, we should consider the possibility that the expression levels or the function

of the *ssrA*-tagged protein differ from those of the naive protein. If protein expression levels could be varied using the *ssrA*-tag, the range of control over intracellular protein levels would need to be further explored. Even in our experiment, the fluorescence of EGFP used in our example was lower in the *ssrA*-tag than in the non-tagged EGFP variant. Third, our system was based on plasmid expression systems. Even in a low-copy-number plasmid, the expression is higher than the genome-derived expression. Therefore, introducing this system in the genome may enable us to investigate more closely resembling physiological functions.

5. Conclusions

The protein degradation system developed in this study has demonstrated the potential to control intracellular protein levels to a certain extent. Further investigation could enable more precise control of protein expression in *V. parahaemolyticus*. Moreover, experimentally controlling intracellular protein levels will allow for a more detailed examination of the relationship between protein quantity and cellular phenotype, potentially overcoming the limitations of the “all-or-nothing” model.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biology15050430/s1>, Table S1: Plasmids and strains used in this study; Table S2: Primers.

Author Contributions: T.U. designed the study, wrote the manuscript, and participated in data analyses and funding acquisition. T.U., K.K., A.M. and H.I. performed biological assays. H.I., M.A., T.S., K.M. and A.T. were involved in the study design, manuscript preparation, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

EGFP	Enhanced Green Fluorescent Protein
LDH	Lactate Dehydrogenase
PBS	Phosphate-buffered Saline
RFU	Relative Fluorescence Unit
TDH	Thermostable Direct Hemolysin
TRH	TDH-Related Hemolysin
T3SS	Type III Secretion System

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