Identification and characterization of a translation arrest motif in VemP by systematic mutational analysis

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VemP (Vibrio protein export monitoring polypeptide) is a secretory protein comprising 159 amino acid residues, which functions as a secretion monitor in Vibrio and regulates expression of the downstream V.secDF2 genes. When VemP export is compromised, its translation specifically undergoes elongation arrest at the position where the Gln¹⁵⁶ codon of vemP encounters the P-site in the translating ribosome, resulting in up-regulation of V.SecDF2 production. Although our previous study suggests that many residues in a highly conserved C-terminal 20-residue region of VemP contribute to its elongation arrest, the exact role of each residue remains unclear. Here, we constructed a reporter system to easily and exactly monitor the in vivo arrest efficiency of VemP. Using this reporter system, we systematically performed a mutational analysis of the 20 residues (His¹³⁸–Phe¹⁵⁷) to identify and characterize the arrest motif. Our results show that 15 residues in the conserved region participate in elongation arrest and that multiple interactions between important residues in VemP and in the interior of the exit tunnel contribute to the elongation arrest of VemP. The arrangement of these important residues induced by specific secondary structures in the ribosomal tunnel is critical for the arrest. Pro scanning analysis of the preceding segment (Met¹²⁰–Phe¹³⁷) revealed a minor role of this region in the arrest. Considering these results, we conclude that the arrest motif in VemP is mainly composed of the highly conserved multiple residues in the C-terminal region.

Newly synthesized non-cytoplasmic proteins must traverse or be integrated into the membrane to localize to their final destinations. In bacteria, both the SecA ATPase, an essential motor protein, and the SecYEG translocon, which forms a transmembrane path for secretory proteins, play central roles in protein translocation (1, 2). In addition, SecDF, a heterodimeric complex of the membrane proteins SecD and SecF, also participates in this event. The Escherichia coli SecDF is required for efficient protein export in vivo and cell growth at low temperatures (3). Based on the crystal structures of SecDF and structure-instructed biochemical and biophysical studies, we previously proposed a model for SecDF-mediated facilitation of protein translocation at a late step in protein export (4, 5). Our data strongly suggest that E. coli SecDF pulls a translocating polypeptide chain from the periplasmic side, coupled with proton flow from the periplasmic space to the cytosol through a SecD-SecF transmembrane interface.

Separately, we also reported that most Vibrio species possess two sets of SecDF paralogs, namely, V.SecDF1 and V.SecDF2, that utilize Na⁺ and H⁺ motive forces, respectively, to stimulate protein translocation (6), providing a physiological role for monovalent cation conductance in SecDF function. Furthermore, our biochemical studies revealed the existence of a unique regulatory mechanism for V.SecDF2 in Vibrio alginolyticus (6). Although expression of the H⁺-driven V.SecDF2 is tightly repressed under normal protein export conditions, it is dramatically up-regulated when protein export is compromised. A u-orf located upstream of the V.secD₂-V.secf₂ (V.secDF2) operon performs a critical role in V.SecDF2 expression. The u-orf encodes a secretory protein, VemP (Vibrio protein export monitoring polypeptide), consisting of 159 amino acid residues, including a C-terminal segment of ~20 amino acid residues that is highly conserved among Vibrio species. Our analysis demonstrates that translation of VemP undergoes elongation arrest near the C-terminal position in response to protein export defects. The stable retention of the ribosome at a specific position on the vemP-V.secDF2 mRNA presumably destabilizes a secondary structure of the vemP-V.secDF2 intergenic region, leading to exposure of the Shine-Dalgarno sequence of V.secD₂ and consequent induction of V.SecDF₂ proteins (6). In contrast, under normal protein export conditions, the elongation arrest of VemP is transient because a pulling force driven by the protein translocase destabilizes the arrested state of VemP (6).

Our biochemical studies using an in vitro translation system with E. coli ribosomes elucidated the arrested state of VemP (6). A toe print assay indicated that the elongation arrest specifically occurs at the position where the Gln¹⁵⁶ codon encounters the P-site in the translating ribosome. Northern blot analysis also showed that the arrested VemP polypeptide contains a Gln-tRNA molecule at the C terminus. Thus, the peptidyl transfer reaction to Phe¹⁵⁷-tRNA in the A-site is blocked. The elongation arrest of VemP also occurred at the same position when a ribosome fraction prepared from the native organism V. alginolyticus was used (6). A systematic stop codon scan and
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Ala/Ser scan analyses using both E. coli and V. alginolyticus ribosomes showed that both ribosomes exhibit the same amino acid specificity for the elongation arrest of VemP. As many as 17 amino acid residues in the C-terminal conserved region contribute to the elongation arrest (6); however, the exact role of each amino acid residue of the conserved region in VemP elongation arrest remains unknown.

Previously, two mutations (G91D and A93T) in rplV, which encodes the ribosomal large subunit protein 22 (L22), were isolated as arrest-defective mutations for SecM (7), the first identified intrinsic arrest polypeptide (7–9). These mutations are located at a constriction site in the ribosome exit tunnel. The former mutation dramatically decreased the VemP arrest efficiency, whereas the latter mutation did not; thus, specific interactions exist between the VemP nascent chain and the interior of the exit tunnel of the ribosome, and the interaction mode of VemP is different from that of SecM (6). However, the mechanism via which the conserved segment inactivates the peptidyl transfer center (PTC)2 of the ribosome remains unclear.

In this study, we constructed and evaluated an E. coli reporter system to easily and precisely monitor the in vivo elongation arrest efficiency of VemP. Using this reporter system, we performed a systematic mutational analysis of the conserved 20-residue segment of VemP to identify and characterize the arrest motif. On the basis of the results of our biochemical experiments and a recently published cryo-EM structure (10), we discuss the mechanism via which the VemP arrest motif interacts with the ribosomal tunnel and stabilizes the elongation arrest state. We also observed that the arrested VemP form undergoes signal-sequence processing. The implications of this finding are also discussed.

Results

Construction of a fusion protein to monitor the in vivo translation elongation arrest of VemP

VemP translation undergoes elongation arrest near its C terminus (6). To distinguish between the fully synthesized VemP polypeptide and the arrested polypeptide by SDS-PAGE after removal of the tRNA moiety, we constructed a plasmid, pTS47, carrying vemP-(flag)3-myc under the control of a lac promoter, in which a DNA fragment encoding the 3× FLAG and Myc tag were fused in frame to the last codon (GCC) of VemP, which encodes Ala159 (Fig. 1A). The tagged protein was expressed in E. coli, and the in vivo kinetics of the release of the VemP-arrested form was examined by pulse-chase experiments. Bands corresponding to the arrested polypeptide with the unprocessed signal sequence (156 residues), the arrested and signal sequence-processed polypeptide (130 residues), and the mature (signal sequence-processed) full-length product (174 residues) were clearly separated by large differences in mobility on SDS-PAGE (Fig. 1B, compare lanes 1–3 and 10–12). In a sec-deficient condition obtained by overexpression of the Syd protein in a secY24 background (11, 12), the unprocessed/arrested products remained stable throughout the chase period.

Requirement of conserved amino acid sequence for elongation arrest of VemP

Previously, we used Ala/Ser scanning of the C-terminal conserved region of VemP and showed that many amino acid residues in this region are involved in elongation arrest (6). However, substitution of an amino acid residue is always accompanied by a nucleotide change at the corresponding codon. To exclude the possibility that the nucleotide sequence of the mRNA, rather than the amino acid sequence, is the primary determinant of translation arrest, we constructed a mutant plasmid that had the same nucleotide sequence (underlined in Fig. 1A) by introducing frameshift mutations (represented as F.S. in Fig. 1, A and B). Specifically, the first base (T) of the codon for Tyr132 (TAT) in the template plasmid pTS47 was deleted, and a T was inserted just before the codon for Ser158 (TCT) (Fig. 1A). Clearly, the cells expressing the frame-shifted protein did not produce any arrested product even in the sec-deficient condition (Fig. 1B, lanes 16–18). We also examined the effects of five synonymous mutations at the Leu153 residue in the conserved region on the elongation arrest efficiency using a new reporter system described below (results are shown in Fig. 3H). All cells carrying plasmids with the synonymous mutations exhibited essentially the same arrest activity as cells harboring the wildtype plasmid. These results indicate that the translation elongation arrest of VemP depends on the synthesized polypeptide sequence.

The abbreviations used are: PTC, peptidyl transfer center; F.S., frameshift; IPTG, isopropyl-β-D-galactopyranoside.
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Although pulse-chase experiments using the VemP-(FLAG)$_3$-Myc proteins allowed us to precisely measure the in vivo stability of the VemP-arrested products, this method is not suitable for simultaneously analyzing a larger number of samples. To systematically characterize a number of VemP derivatives, we constructed a reporter system to easily monitor the stability of VemP’s ability to arrest its own translation elongation by measuring LacZ enzymatic activity (Fig. 2A). The reporter plasmid pHM1144 carries the ss-vemP-lacZ fusion gene under the control of a lac promoter, in which the codon for Ile4 of LacZ (ATT) is fused in frame to the codon for Ser158 of VemP (TCG) (Fig. 2A). We expected that translation of the fusion protein would be stably halted at Gln156, the arrest point of VemP. In vivo analysis of VemP elongation arrest. A, schematic structure of VemP-(FLAG)$_3$-Myc. Partial nucleotide sequences of the plasmids used in this study are shown. WT, ΔC, and F.S., wildtype, mutant deleted C-terminal segment, and frame-shifted mutant, respectively. Boldface and italic type represent the PstI restriction site and part of the FLAG sequence, respectively. Deduced amino acid sequences are shown at the bottom. The boxed and underlined amino acid sequences indicate the highly conserved motif in VemP and the amino acid sequence in the corresponding region of the F.S. mutant, respectively. B, in vivo kinetics of the release of elongation arrest and the processing of VemP. Wildtype cells with vector (pSTV28) and secY24 mutant cells with psyd were transformed with plasmids encoding the VemP-(FLAG)$_3$-Myc derivatives as indicated. The cells were grown in an M9-based medium until an early log phase at 30 °C, 1 mM IPTG, and 2 mM cAMP were added, and cells were further cultivated for 30 min at 30 °C to produce both VemP derivatives and Syd. The cultures were then labeled with [35S]methionine for 30 s, followed by chase reaction by the addition of excess amounts of cold methionine for the indicated time. Labeled VemP derivatives were immunoprecipitated with anti-VemP antibody. Arrested bands (the unprocessed (un) and processed (pro) bands), and full-length products (the precursor (p) and mature (m) bands) are indicated. The arrested and full-length products of the wildtype VemP-(FLAG)$_3$-Myc in the Sec/WT cells (lanes 1–3) and Sec/ΔC cells (lanes 10–12) were quantified. The calculated arrest efficiencies of the samples with S.D. (n = 2) are shown graphically in Fig. S1A. C, generation of processed/arrested form of VemP. Wildtype cells were transformed with a plasmid encoding either the VemP-(FLAG)$_3$-Myc or SecM-(FLAG)$_3$-Myc, as indicated. The cells were grown in an M9-based medium until an early log phase at 30 °C. Then the cultures were divided into two portions, and 1 mM IPTG and 2 mM cAMP were added to each. They were further cultivated for 15 min at 30 °C to produce either VemP-(FLAG)$_3$-Myc (lanes 1–6) or SecM-(FLAG)$_3$-Myc (lanes 7–12). 10 min after the addition of the inducers, 3 mM NaN$_3$ was added only to one of the two cultures (lanes 4–6 and 10–12) to inactivate SecA ATPase. The cultures were then labeled with [35S]methionine for 30 s, followed by chase for the indicated time. Labeled MBP and OmpA were immunoprecipitated with a mixture of anti-MBP and anti-OmpA antibodies, separated by 10% SDS-PAGE, and analyzed by phosphorimaging (top gel). The precursor (p) and mature (m) bands of MBP and OmpA are indicated. The calculated translocation efficiencies of MBP and OmpA in the samples with S.D. (n = 2) are shown as the top graph of Fig. S1B. Separately, labeled VemP and SecM were immunoprecipitated with anti-VemP and anti-SecM antibodies, respectively, separated by 10% neutral pH SDS-PAGE, and detected by phosphorimaging (bottom gel). Individual bands were marked accordingly as mentioned above. The calculated arrest efficiencies of the samples with S.D. (n = 2) are shown as the bottom graph of Fig. S1B.
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Figure 2. Construction and validation of the ΔSS-VemP·'·LacZ reporter system. A, schematic structure of ΔSS-VemP·'·LacZ. The nucleotide and deduced amino acid sequences of the junction between vemP and lacZ genes are shown. The underlined segment indicates the BamHI restriction site used for plasmid construction. B, relative LacZ activity of cells carrying reporter plasmids with mutations. LacZ activity of JM109 cells expressing the indicated ΔSS-VemP·'·LacZ derivatives was measured and expressed relative to the wildtype with S.D. (error bars) (n = 2). C, abundance of ΔSS-VemP·'·LacZ proteins. Proteins in the cells used in Fig. 2B were precipitated with 5% trichloroacetic acid and solubilized with 2% SDS, followed by RNase treatment (+) or mock treatment (−). The VemP-arrested products and LacZ fusion proteins were then separated by 10% neutral pH SDS-PAGE and detected by immunoblotting with anti-VemP antibody. MWM, molecular weight markers. D, relative LacZ activity of Ala/Ser mutants. The LacZ activity of E. coli JM109 cells expressing the ΔSS-VemP·'·LacZ derivatives with single Ala/Ser mutations (indicated) in the conserved region was measured, and activity relative to the wildtype is shown with S.D. (n = 2). The mutants were classified into five groups as shown on the right side of the graph, based on the extent of LacZ activity. The same classifications were applied to the results of the systematic mutational analysis in Fig. 4. The numbers at the bottom of the graph show in vitro arrest efficiencies of the Ala/Ser mutants, which are transcribed from the data of Fig. 4E from our previous publication (6). N.D., not determined. E, pulse-chase experiments of VemP-(FLAG)3-Myc derivatives. The Ala/Ser mutations indicated at the bottom of the gel were introduced into the plasmid shown in Fig. 1A. Protein export-deficient cells (secY24/pSyd) expressing VemP-(FLAG)3-Myc derivatives were pulse-chased and analyzed according to the legend of Fig. 1B. Bands representing precursor (p) and mature (m) forms of full-length product and arrested/unprocessed forms (arrest) are shown. Arrest efficiency at each time point was calculated as arrested band/(arrested band + precursor band) and used in Fig. 2F. F, quantified results of the pulse-chase experiment. Average values of arrest efficiency with S.D. (n = 2) are plotted against chase time. G, correlation between elongation arrest and LacZ activity. The percentage arrest values of the Ala/Ser mutants at 0.5 min in Fig. 2F were plotted against relative LacZ activity of the corresponding mutants shown in Fig. 2D.

of VemP, because of the deletion of the signal sequence that is essential for protein translocation (and consequent arrest release), resulting in low LacZ activity. In contrast, synthesis of the fusion protein with a mutation in the conserved region required for elongation arrest resulted in continued elongation until the C-terminal end and high LacZ activity.

First, the reporter system was validated using control constructs encoding the ΔSS-VemP(ΔC)·'·LacZ and ΔSS-
VemP(F.S.)-’LacZ proteins, both of which harbored the same mutations shown in Fig. 1. As expected, E. coli expressing the ΔC or the F.S. derivative exhibited -15-fold higher LacZ activity than cells expressing the "wildtype" reporter (Fig. 2B). On neutral pH SDS-PAGE, the cells expressing wildtype ΔSS-VemP-’LacZ showed a distinct 35-kDa product that cross-reacted with anti-VemP antibody and was converted to a 15-kDa ΔSS-VemP polypeptide fragment by RNase treatment (Fig. 2C, lane 1 versus lane 2), indicating that the 35 kDa band was an arrested product containing a tRNA moiety. In contrast, E. coli cells expressing ΔSS-VemP(ΔC)-’LacZ or ΔSS-VemP(F.S.)-’LacZ did not generate such a peptidyl-tRNA product but produced large amounts of the full-length fusion protein (Fig. 2C, lanes 3–6). Based on the assumption that the stability of the mutant proteins was not significantly altered by the mutations, we speculated that the 15-fold higher LacZ activity relative to the wildtype represents the maximum value that can be obtained under the assay conditions, which results from repeated cycles of translation of the arrest-defective VemP derivatives from the individual mRNAs.

Next, we introduced a series of Ala/Ser mutations in the reporter plasmid and measured the LacZ activities of cells expressing the ΔSS-VemP-’LacZ mutant proteins (Fig. 2D). The results clearly show an inverse correlation between LacZ activity (Fig. 2D, graph) and in vitro production of VemP-arrested products with these Ala/Ser mutants; the in vitro percentage in Fig. 2D were transcribed from the data of the in vitro arrest efficiency experiment shown in Fig. 4E of our previous report (6). Cells expressing mutated proteins with Ala/Ser substitutions that reduced in vitro elongation arrest efficiency had >8-fold higher LacZ activity than cells expressing the wildtype reporter in most cases, suggesting that single Ala substitutions in the C-terminal conserved region considerably reduced the elongation arrest ability in vivo.

To further investigate whether the increase in relative LacZ activity observed in the mutants reflects the extent of reduction of their arrest efficiency, we introduced six mutations, A148S, G142A, E145A, I140A, W143A, and Y150A, which resulted in different levels of LacZ activity (Fig. 2D), into the plasmid encoding VemP-(FLAG)3-Myc and measured the stability of these arrested products by pulse-chase experiments under Sec-deficient conditions (Fig. 2, E and F). The arrest efficiency of the mutants observed at 0.5 min after initiation of the chase reaction (Fig. 2F) were plotted against the relative LacZ activity of the mutants shown in Fig. 2D. As shown in Fig. 2G, the arrest efficiency was inversely correlated with LacZ activity, indicating that the translation elongation arrest ability of VemP derivatives can be estimated semiquantitatively from the LacZ activity of the reporter system. The reporter assay using the Ala/Ser derivatives in ribosomal mutant cells that affected the arrest efficiency of VemP also supported the utility of the assay system (Fig. S3 and supporting Results).

**Systematic mutational analysis of the highly conserved C-terminal region of VemP**

Using the plasmid pHM1144 encoding ΔSS-VemP-’LacZ as a template, we systematically constructed 380 plasmids in which each amino acid residue from His138 to Phe157 was replaced with the other 19 amino acid residues (20 residues × 19 amino acids). A list of the constructed plasmids is shown in Table S3. First, we prepared five master microtiter plates (number 1 for residues His138–Ser141, number 2 for Gly142–Glu145, number 3 for Thr146–Met149, number 4 for Tyr150–Leu153, and number 5 for Asn154–Phe157; see the top of Table S3), each of which contained four sets of 20 types of cells with a different plasmid encoding the wildtype fusion protein or the other 19 amino acid alterations of a target residue. To secure the reliability and reproducibility of the extensive experimental data, we cultivated all of the cells in a master plate at the same time in a 96-well deep-well microtiter plate using an M. BR-034P air shaker (Taitec Co. Ltd.) as reported previously (18). The cultures expressing the fusion proteins were used for the LacZ assay (see “Experimental procedures”).

As described above, E. coli cells with the wildtype plasmid were grown at four different positions in each master plate. In all experiments, the S.D. of the LacZ activity obtained from the four wildtype cell types was <14% of the mean (the average S.D. was ~7%), indicating that the control cells exhibited similar LacZ activity irrespective of the position on the plate at which the cells were placed. Thus, we concluded that the assay procedure was highly reproducible and hence suitable for systematic analysis. The LacZ activity of individual cells was measured independently at least twice and normalized to the average value of the wildtype. The results with S.D. are summarized in Fig. 3 (A–E), which shows the relative LacZ activity of the ΔSS-VemP-’LacZ derivatives with replacement of the target residue (top left) with the amino acids indicated at the bottom of the graph.

Replacement of any of the amino acids in the His138–Phe157 region resulted in increased LacZ activity (defined as a <3-fold increase). For all residues other than His138, Ala152, Ser155, Gln156, and Phe157, replacement with a number of individual amino acids considerably increased LacZ activity. Interestingly, although few mutations, such as T146M, exhibited close to maximal activity (~15-fold higher than that of the wildtype), the LacZ activity after introducing the point mutations at most positions was ~10 times that observed with the wildtype and significantly lower than the maximal value.

To gain an overview of the relative LacZ activity of each mutant, we classified the mutants into five groups based on the relative LacZ activity, represented by the gradient in blue color at the right side of Fig. 2D. The data are presented as a matrix in Fig. 4. The horizontal and vertical lines show the amino acid sequence (His138–Phe157) of the VemP conserved region and the amino acid residues to which the target residues are altered, respectively. More than three-fourths of the single mutations at the Trp143 (17 mutations), Lys144 (16), Asn147 (17), Tyr150 (15), and Leu153 (15) residues increased LacZ activity by at least 8-fold. For these residues, only a small number of cognate mutations, such as Y150F and L153I, did not significantly affect LacZ activity. In contrast, more than half of the single mutations at the His138 (15 mutations), Ala152 (12), Ser155 (15), Gly156 (12), and Phe157 (13) residues exhibited a <5-fold increase in LacZ activity over the wildtype, showing that these residues are relatively tolerant to amino acid alterations. The three residues Ile140, Gly142, and Met149 and the Ser141 residue...
tolerated alteration to hydrophobic and hydrophilic residues, respectively. No obvious tendency was observed regarding the effects of alterations at the Glu$^{145}$, Ala$^{148}$, or Val$^{151}$ residues on LacZ activity.

**Statistical analysis of mutation data**

To statistically understand the tolerance to amino acid substitutions for each residue, we represented the obtained LacZ data as box-and-whisker plots (Fig. 5A) showing the LacZ activity relative to that of the wildtype for the 19 mutations of each target residue. Based on the median value (the white bar in Fig. 5A) of the LacZ activity for each residue, we grouped the targeted residues into three classes: most sensitive (shown in blue in Fig. 5 (B and C)), moderately sensitive (green), and relatively resistant to amino acid alterations. The Arg$^{139}$, Trp$^{143}$, Lys$^{144}$, Thr$^{146}$, Asn$^{147}$, Tyr$^{150}$, Leu$^{153}$, and Asn$^{154}$ residues belonged to the first group, with $>8$-fold higher LacZ activity relative to the wildtype. We further divided this group into two subgroups, one (Trp$^{143}$, Lys$^{144}$, and Asn$^{147}$) consisting of residues that did not accept any other residue at their positions and the other (Arg$^{139}$, Thr$^{146}$, Tyr$^{150}$, Leu$^{153}$, and Asn$^{154}$) consisting of residues that exhibited partial tolerance to certain mutations, as judged from the width of the individual black boxes and whiskers that reflect variation in LacZ activity resulting from mutations at individual residues. The Ile$^{140}$, Ser$^{141}$, Gly$^{142}$, Glu$^{145}$, Ala$^{148}$, Met$^{149}$, and Val$^{151}$ residues were classified in the second group, with mutations resulting in an intermediate increase in

**Figure 3. LacZ activity of VemP mutants relative to wildtype.** LacZ activity of cells expressing the ΔSS-VemP∗-LacZ protein, in which the residue shown at the top left of each graph was substituted with the residue indicated along the horizontal axis, was measured twice. The average LacZ activity of the mutants relative to that of the wildtype is shown with S.D. (error bars). A–E, LacZ activity of cells expressing single amino acid deletions (F), Pro mutants (G), or synonymous mutations at Leu$^{153}$ (H) was also measured and expressed relative to that of the wildtype with S.D.
LacZ activity. The His$^{138}$, Ala$^{152}$, Ser$^{155}$, Gln$^{156}$, and Phe$^{157}$ residues were categorized in the third group, with mutations resulting in a slight but significant increase in LacZ activity.

Intriguingly, a periodic fluctuation in the median values after every three or four residues was observed in the C-terminal half of the analyzed region (from Asn$^{147}$ to Ser$^{155}$). In contrast, the N-terminal half of the region (Arg$^{139}$–Thr$^{146}$) showed relatively higher median values. Several residues (Ser$^{155}$–Phe$^{157}$) close to the arrest point (Gln$^{156}$) seemed to be relatively tolerant to most mutations.

Replacement of all residues except for Phe$^{157}$ with Pro was found to significantly enhance LacZ activity (Figs. 4 and 5B). We also observed that replacement of Phe$^{157}$ with Lys dramatically increased LacZ activity (Fig. 5A).

**Single amino acid deletion analysis of the VemP conserved region**

We next determined whether the precise locations of the amino acid residues in the conserved region were important for elongation arrest using a deletion analysis. We selected the four residues Ser$^{141}$, Ala$^{148}$, Ala$^{152}$, and Ser$^{155}$ as targets because these residues were relatively tolerant of amino acid replacements compared with other residues (Figs. 4 and 5A) and because they lie between residues whose mutations strongly impaired the arrest (Arg$^{139}$, Trp$^{143}$, Lys$^{144}$, Tyr$^{150}$, Leu$^{153}$, and Asn$^{154}$) and the arrest point (Gln$^{156}$). All four deletion mutants exhibited >8-fold increase in LacZ activity (Fig. 3F and the uppermost part of the matrix in Fig. 4), indicating that they all compromised elongation arrest.

**Pro scanning analysis of the N-terminal portion of the VemP conserved region**

Sequence alignment of VemP proteins from 42 *Vibrio* species indicated that the C-terminal portion (His$^{138}$–Phe$^{157}$) is highly conserved, whereas the homology of the more N-terminal segment is limited (Fig. 5C and Table S4). As described above, Pro substitution of the target residues appeared to be a good probe for evaluating the importance of the residues. We performed Pro scanning mutagenesis of 18 residues from Met$^{120}$ to Asp$^{137}$ to extend our analysis to the N-terminal portion, as a recent publication on VemP structure (10) suggested that this region could contribute to the elongation arrest of VemP (see “Discussion”). Compared with the results obtained with Pro mutations in the conserved region, most Pro mutants had little effect on LacZ activity (Fig. 3G and the bottom of Fig. 4), although Pro substitutions of Phe$^{131}$ and His$^{133}$ slightly increased LacZ activity.
tein. We observed that the arrested/unprocessed form of VemP remained stable when the translocation machinery was inactivated (Fig. 1(B and C) and Fig. S1). Our results indicate that an ongoing protein translocation reaction is required to resume peptide elongation of the arrested form of VemP (6). Interestingly, we also noticed that under normal protein export conditions, processing of the signal sequence by leader peptidase mostly occurs before release from the arrested state, with gradual conversion of the processed/arrested product to the full-length mature form (Fig. 1, B and C). Our results strongly suggest that the VemP peptide remains in the elongation-arrested state at the early stages of its translocation through the SecYEG channel and that peptide elongation restarts, perhaps triggered by a force provided at a later stage during translocation. These findings suggest that the force generated by the SecA ATPase to push the nascent VemP into the membrane (19, 20) is either not sufficient or not used for the release of the VemP-arrested state and that a pulling force at a later step, such as the SecDF-promoted release into the periplasmic space (5), is required for resumption of peptide elongation. Because VemP monitoring of the cellular activity of protein export up-regulates V.SecDF2 proteins in the native organism V. alginolyticus (6), the elongation-arrested state of VemP at the early stages of its membrane translocation appears to be well-suited to sense the functional state of SecDF that acts at the early to late stages of protein translocation. In contrast, a processed yet arrested product was not observed for SecM (Fig. 1C)(8, 16, 17). Because SecM regulates the expression of SecA ATPase (8, 21), its expression may be optimized to monitor SecA function at the early stages of translocation. However, further detailed study is required to understand the physiological significance of the arrested/processed state of VemP. In particular, the molecular basis of sensing different substeps of translocation by various regulatory nascent chains is an interesting subject for future studies.

The VemP arrest motif

We also constructed a ΔSS-VemP‘-LacZ reporter system and performed a systematic mutational analysis of the 20 residues in the C-terminal conserved segment of VemP by monitoring the in vivo arrest activity. Although the results of the statistical analysis (Fig. 5A) roughly corroborated those of a previous Ala/Ser scanning study (6), we observed that the importance of the Ser141 residue had been underestimated by

Figure 5. Statistical analysis of the results of the reporter assays. A, box-and-whisker plots of relative LacZ activity. Relative LacZ activities of 19 single amino acid mutants at each target residue are presented as box-and-whisker plots. The top and bottom ends of the black boxes show the upper (Q3/4) and lower (Q1/4) quartiles of the LacZ activities, respectively. The white bars indicate the median values. The top and bottom ends of the whiskers show the highest datum within Q3/4−1.5×(Q3/4−Q1/4) and the lowest datum within Q1/4+1.5×(Q3/4−Q1/4), respectively. Outliers outside these regions are shown as circles with single-letter representations of the amino acid to which the target residue was mutated. B, Pro scanning analysis. Relative LacZ activities of the Pro mutants are shown. C, amino acid sequence conservation of the C-terminal region of VemP. A sequence logo was generated by WebLogo (http://weblogo.threeplusone.com/) using amino acid sequences of the C-terminal 38 residues from 42 VemP homologs among Vibrio species (all sequence data are shown in Table S4). Numbers indicate amino acid positions in V. alginolyticus 138-2 VemP. The highly important residues and important residues determined in this study are indicated by blue and green inverted triangles, respectively.
the cognate substitution from Ser to Ala in the previous study (Fig. 2D). The statistical data clearly show that numerous amino acid residues contribute to the elongation arrest of VemP. In particular, the Trp^{143}, Lys^{144}, Tyr^{150}, Leu^{153}, and Asn^{154} residues seem to be extremely important. Because the four single deletions (Fig. 3F) and almost all of the Pro substitutions (Fig. 5B), which are predicted to destabilize the secondary structure of the polypeptide, largely compromise VemP elongation arrest, the secondary structure–induced spatial arrangement of the important residues in the ribosome tunnel is likely to be crucial for the arrest activity of VemP. It is noteworthy that although various single mutations can considerably decrease the elongation arrest activity of VemP, these mutations individually cannot completely abolish the arrest activity (compare Fig. 2B with Fig. 3 (A–E)), suggesting that none of the individual contacts between the VemP nascent chain and the interior regions of the translating ribosome is essential for the elongation arrest. Probably, multiple interactions contribute to the stability of elongation arrest. In contrast, the His^{138}, Ala^{152}, Ser^{155}, Gln^{156}, and Phe^{157} residues play apparently minor roles in VemP elongation arrest.

The periodic appearance of functionally important residues in the latter half of the conserved region (Asn^{147}–Ser^{153}) and the extreme sensitivity of the Pro alterations in the region support the idea that the region forms an α-helix, of which one surface specifically interacts with ribosomal components in the exit tunnel. This prediction is supported by the VemP-ribosome structure published recently (10) (see below). The former half of the conserved region (Arg^{139}–Thr^{146}) also appears to exhibit a weak periodic profile for functionally important residues (Fig. 5A). However, most of the amino acid residues in the former half of the region are crucial for elongation arrest. The compact and unique secondary structure of the region observed on cryo-EM (10) may explain why these functionally important residues are concentrated in the region (see below).

Pro scanning analysis of the N-terminal region (Met^{120}–Asp^{137}) of the conserved segment (Fig. 3G and the bottom of the matrix in Fig. 4) showed that the individual residues in the region play a minor role in VemP elongation arrest, which is consistent with the lower conservation of this region among Vibrio species (Fig. 5C and Table S4). Needless to say, we cannot exclude the possibility that some features of the preceding region, such as α-helix formation, may partially contribute to the stability of elongation arrest. Taking these results together, we propose that a number of amino acids in the C-terminal conserved segment constitute the arrest motif of VemP.

It should be noted that in the current study, we analyzed the importance of individual amino acid residues in VemP elongation arrest in E. coli; the contributions of the individual residues in the conserved motif to VemP arrest may not necessarily be equal to those in the native organism V. alginolyticus. Indeed, studies show that SecM and MifM exhibited translational arrest when they were synthesized with the cognate ribosomes (E. coli and Bacillus subtilis, respectively) in vitro but did not in heterologous combinations (22). A recent study on the structure and sequence conservation of the E. coli and B. subtilis ribosomes revealed that the rRNA regions located on the interior surface of ribosome tunnel and the luminal part of the L4 protein are highly conserved between these species, but a β-hairpin of L22 is variable (23). Mutational analyses suggested that a single amino acid in the β-hairpin of the L22 dictates the species specificity of the arrest event (23). On the other hand, E. coli and V. alginolyticus are closely related species belonging to γ-proteobacteria. More than 80% of the amino acid residues of L4 and L22 are identical between the two organisms, and furthermore, the amino acid sequences of the β-hairpin of L22 are almost completely conserved. Thus, we expect that the results pertaining to VemP arrest observed in the E. coli system might reflect the mode of VemP arrest in its native organism V. alginolyticus, although it should be validated experimentally. In any case, our results would provide clues for understanding the molecular mechanisms of translational arrest.

**Structural basis of the functionally important residues for VemP arrest**

The cryo-EM structure of the VemP-ribosome complex at a resolution of 2.9 Å was reported during completion of our systematic mutational analysis (10). The VemP-ribosome structure revealed that the arrested VemP polypeptide is in an extremely compact conformational state in the ribosome exit tunnel (shown as the gray tube model in Fig. 6A). Intriguingly, the 37 residues of the Met^{120}–Gln^{153} region of VemP form two α-helices (Met^{120}–Ser^{129} and Thr^{146}–Gln^{156}) connected by an α-turn and loop. According to the structure of the VemP-ribosome complex (Protein Data Bank entry 5NWY) (Fig. 6A), the Tyr^{150} (in red) and Asn^{154} (purple) residues, both of which are crucial for VemP arrest, are located on the same surface of the upper α-helix close to the PTC and specifically interact with C2610 (in red) and both U2506 (in the b conformational state) and U2584 of the 23S RNA (in purple), respectively. The existence of π–π interactions between the Tyr^{150} residue of VemP and the C2610 of the 23S rRNA (Fig. 6A) is consistent with the weak effect of the Y150F substitution on LacZ activity (Figs. 4 and 5A). Although not pointed out by Su et al. (10), the highly important Asn^{147} residue (in deep green) also interacts with both G2505 and C2611 of the 23S rRNA (deep green) (also see Fig. 5A). In contrast, a close inspection of distances between side chains of the VemP polypeptide and ribosomal residues in the cryo-EM structure (Table S5) did not reveal any predictable interaction between ribosomal components and the residues located on the opposite surface of the upper α-helix (Ala^{152}, Ser^{155}, and Gln^{156}). These results fit well with the periodic appearance of the functionally important residues described above.

The side chains of the Arg^{139} (in green in Fig. 6A), Trp^{143} (pale blue), and Lys^{144} (blue) residues specifically interact with A752 (in green) of the 23S rRNA, the Arg^{157} residue (pale blue) of the L22 subunit, and U747 (blue) of the 23S rRNA, respectively. The extremely compact packing of the region near the ribosomal constriction site (Arg^{139}–Thr^{146}) may be a reason for the strict requirement for the residues at this site. An interaction between the His^{133} residue (in yellow) of VemP and Lys^{58} (also yellow) of the L4 subunit was observed in the cryo-EM structure (10). This is consistent with our finding that the H133P mutation weakly but significantly increases LacZ activity by ~3-fold.
Thus, the structural data and our biochemical data are comprehensively and mutually supportive.

However, close inspection of the VemP-ribosome structure (Table S5) revealed no predictable interactions of the other crucial residues (Thr$^{146}$ and Leu$^{153}$, in blue in Fig. 6B) or the important residues (Ile$^{140}$, Ser$^{141}$, Glu$^{145}$, Ala$^{148}$, Met$^{149}$, and Val$^{151}$, in green) with ribosomal components. Of course, we expect that certain mutations (such as ones from a small side chain to a large one) in these residues would inhibit the formation of the specific secondary structure of VemP in the exit tunnel due to steric hindrance of the introduced side chain that might interfere with the formation of the hydrogen-bonded network. Intriguingly, except for His$^{133}$, the functionally important residues, with (Fig. 6A) or without (Fig. 6B) apparent interactions with ribosomal components, exhibit a biased localization to the same side of the ribosome tunnel. Thus, we can also conceive that some residues positively contributed to the elongation arrest of VemP via direct interactions with ribosomal residues located at the same hemisurface of the tunnel during establishment of the VemP-arrested state.

Whereas the reported structure represents a snapshot of the arrested state of VemP, we assume that different conformational states of VemP exist before the stable arrested state is established. For forming the VemP-arrested state, first, a portion of the elongating VemP polypeptide may specifically contact some ribosomal residues in the exit tunnel, resulting in a transient stop in the peptide movement toward the tunnel exit. The interruption in the movement at the contact site and continuous peptide elongation at the PTC allow the VemP polypeptide to form a compact structure and generate further contacts with other components of the ribosome and/or intramolecularly form the specific secondary structure required to inactivate PTC. During these steps, VemP residues, such as Thr$^{146}$, Leu$^{153}$, and some others, may contribute to the stabilization of intermediate states formed before the formation of the final stably arrested state. In this manner, formation of the compact structure of the C-terminal region may require multiple, intratunnel molecular interactions, explaining why many amino acid residues contribute to establishment of the elongation arrest of VemP. Compared with VemP, fewer amino acid residues contribute to the elongation arrest of other arrest polypeptides, such as SecM (7, 24) and MifM (25). The findings that these arrested nascent chains exhibit relatively extended structures in the ribosome tunnel (23, 26, 27) support the notion discussed above. To understand in detail how the VemP-arrested state is established, further studies including molecular dynamic simulations are required.

Potential role of aminoacyl-tRNA at the A-site in the translation arrest in VemP

SecM translation undergoes elongation arrest when the Gly$^{165}$ codon encounters the P-site of the ribosome (28). The finding that the Pro$^{166}$ residue, which is not incorporated into the arrested peptide, is crucial for the elongation arrest (7, 28) strongly suggests that the Pro-tRNA exists in the A-site of the stalled ribosome. This concept is supported by the cryo-EM structure of the SecM-ribosome complex (26). In contrast, replacement of the Phe$^{157}$ codon with a stop codon maintains the VemP-arrested state at the same position, indicating that the VemP peptide potentially has termination arrest activity (6). In this study, we also identified several mutations of Phe$^{157}$ that had little or no effect on elongation arrest (Figs. 4 and 5).
The simplest explanation for these results is that the VemP-stalled ribosome undergoes a conformational change that prevents any other aminoacyl-tRNAs or release factors from binding to the A-site. Consistent with this idea, the VemP-ribosome structure suggests that the interactions between the Asn"Thr residue and both U2585 and U2506 of the 23S RNA cause a conformational change in the VemP-translating ribosome to inhibit accommodation of Phe-tRNA at the A-site (10). However, our data clearly show that the F157K substitution severely compromises VemP elongation arrest (Figs. 4 and 5A). Thus, at least the Lys-tRNA can enter the A-site of the VemP-translating ribosome with unimpaired ability to receive peptide transfer. Additionally, some other substitutions of Phe"Thr significantly, but significantly, decreased the elongation arrest of VemP (Fig. 3E). These new results may indicate that the VemP-stalled ribosome is not robustly inactivated but retains flexibility to cooperate with or be activated by Lys-tRNA and some other aminoacyl-tRNAs. It is also conceivable that the VemP-ribosome complexes are intrinsically dynamic even in the arrested state, as reported for the SecM-ribosome complex (26, 27). Possibly, the arrested ribosome contains Phe-tRNA at the A-site, whereas the peptide transfer reaction is inactivated transiently by the VemP-ribosome interactions. This situation may be advantageous, because physiologically, the elongation arrest must be canceled rapidly as the VemP nascent chain engages in the Sec-pathway translocation. Whether the Phe-tRNA exists at the A-site of the arrested ribosome is an important subject of future studies.

**Experimental procedures**

**Media**

L broth–rich medium (13) and M9 synthetic medium (without CaCl₂) supplemented with maltose (final 0.2%), glycerol (final 0.4%), and 18 amino acids (except Met and Cys; final concentration of 20 µg/ml each) (13) were used for cultivation of E. coli strains. Antibiotics were used at the following concentrations: ampicillin, 50 µg/ml; chloramphenicol, 20 µg/ml.

**Chemicals**

Super high-grade chemical reagents purchased from Sigma-Aldrich or Nacalai Tesque were used for all of the experiments. Restriction endonucleases (New England Biolabs) and the Ligatron High version 2 DNA ligation kit (Toyobo Co., Ltd.) were used for cloning experiments. Prime Star max DNA polymerase High version 2 DNA ligation kit (Toyobo Co., Ltd.) were used for site-directed mutagenesis reaction. The BigDye® Terminator chemically modified DNA analyzer (Thermo Fisher Scientific, Inc.) was used for DNA sequencing.

**Antibody**

The unfractionated anti-VemP serum and the affinity-purified anti-VemP antibody (6) were used for immunoprecipitation and immunoblotting, respectively. Anti-OmpA (29), anti-MBP (14), and anti-SecM (8) were described previously.

**Strains and plasmids**

The E. coli strains used in this study are listed in Table S1. They were constructed as follows. HM4109 (HM1742, secY24 rpsE) and HM4903 (HM1742, secY24 rpsE) were constructed by joint transduction of secY24 and secY39 mutations with zhd-33::Tn10 from TYE055 (30) and SH470 (31) to HM1742, respectively. HM4884 (HM1742, secD1) was constructed by co-transduction of the secD1 mutation to HM1742 with a linked Tn10. HM4911 (HM1742, ΔdegP-kan) was constructed by transducing ΔdegP-kan from JW0157 (32) to HM1742 (33). The plasmids used in this study are also listed in Tables S2 and S3. Nucleotide sequences were determined by Prism3130 DNA analyzer (Thermo Fisher Scientific, Inc.). We confirmed all of the relevant DNA segments that were subjected to *in vitro* replication by sequencing the final constructs.

**lacZ assay**

LacZ activity of the cells expressing ΔSS-VemP"LacZ was measured basically according to the procedure published previously (18). The standard procedure is as follows. 12 µl of fully grown culture was inoculated into 1.2 ml of L medium supplemented with ampicillin in a deep 96-well microtiter plate, and the cultures were shaken at 37 °C for 2.5 h with 1300 rpm using a DWMax BP-304P shaker (Taito co., Japan). Then the culture cells were further cultivated for 30 min in the presence of 10 mM IPTG to induce the ΔSS-VemP"LacZ derivatives. A portion (80 µl) of the cultures was taken and placed in a clear 96-well plate containing 20 µl of Reporter Lysis buffer (Promega, Madison, WI). The cells were lysed by freezing at −80 °C and subsequent thawing at 37 °C for ~20 min. After the addition of 100 µl of 1.32 mg/ml 2-nitrophenyl β-d-galactopyranoside (Sigma-Aldrich) in Z-buffer to each well, samples were mixed vigorously and incubated for 40 min at 25 °C. During this incubation, absorbance at 420 and 550 nm were measured at every 2 min for each well using the Viento Nano microplate reader (BioTek Instruments, Inc.). β-Galactosidase activity in arbitrary units was calculated according to the equation, LacZ activity (arbitrary units) = (A420 − 1.75 × A550)/(incubation time (min) at 25 °C) × (A600 of the bacterial culture at the time of collection)) (34).

**Protein export assay**

An *in vivo* protein export assay was carried out according to the protocol reported previously (35).

**Author contributions**—H. M. and Y. A. conceived the idea and designed the experiments; H. M., S. S., J. I., and E. I. performed the experiments; H. M., E. I., and Y. A. analyzed the data; and H. M. and Y. A. wrote the manuscript.

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