Nascent chain-monitored remodeling of the Sec machinery for salinity adaptation of marine bacteria

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SecDF interacts with the SecYEG translocon in bacteria and enhances protein export in a proton-motive-force-dependent manner. *Vibrio alginolyticus*, a marine-estuarine bacterium, contains two SecDF paralogs, V.SecDF1 and V.SecDF2. Here, we show that the export-enhancing function of V.SecDF1 requires Na^+ instead of H^+, whereas V.SecDF2 is Na^+-independent, presumably requiring H^+. In accord with the cation-preference difference, V.SecDF2 was only expressed under limited Na^+ concentrations whereas V.SecDF1 was constitutive. However, it is not the decreased concentration of Na^+ per se that the bacterium senses to up-regulate the V.SecDF2 expression, because marked up-regulation of the V.SecDF2 synthesis was observed irrespective of Na^+ concentrations under certain genetic/physiological conditions: (*i*) when the $secDF1_{VA}$ gene was deleted and (*ii*) whenever the Sec export machinery was inhibited. VemP (*Vibrio* export monitoring polypeptide), a secretory polypeptide encoded by the upstream ORF of $secDF2_{VA}$, plays the primary role in this regulation by undergoing regulated translational elongation arrest, which leads to unfolding of the Shine–Dalgarno sequence for translation of $secDF2_{VA}$. Genetic analysis of *V. alginolyticus* established that the VemP-mediated regulation of SecDF2 is essential for the survival of this marine bacterium in low-salinity environments. These results reveal that a class of marine bacteria exploits nascent-chain ribosome interactions to optimize their protein export pathways to propagate efficiently under different ionic environments that they face in their life cycles.

protein export | proton-motive force | ribosome | SecD | translation arrest

Cells must export specific proteins to their surfaces for growth, reproduction, and interaction with the environment. In bacteria, the SecA ATPase and the SecYEG translocon play central roles in translocation of secretory proteins across the cytoplasmic membrane (1, 2). In addition, efficient operation of protein export in bacteria requires SecDF, a membrane protein complex having 12 transmembrane segments and large periplasmic domains of functional importance (3). SecDF is encoded either by the separate *secD* and *secF* genes (4) or as a single polypeptide (5). The crystal structures of *Thermus thermophilus* SecDF (TSecDF) and structure-instructed functional studies of the *Escherichia coli* ortholog suggest that SecDF drives polypeptide translocation by capturing a substrate that is emerging from the translocon into the periplasmic space and undergoing proton-motive-force-dependent cycles of conformational changes (5, 6).

In the present study, we address the functions and expression regulation of the two SecDF paralogs in a halophilic, marine-estuarine bacterium, *Vibrio alginolyticus*. The *Vibrio* group of gram-negative bacteria such as *Vibrio parahaemolyticus* are important for human health because many of them cause severe foodborne illness and wound infections (7, 8). *V. alginolyticus* was reported to be involved in toxin production in infected sea animals (9). These bacteria living in seawater, including estuary areas, encounter, migrate into, or are brought by associated animals to different salinity environments. Upon infection to animals, they are also exposed to intrabody and even intracellular fluid (7, 10), whose Na^+ concentrations may be different from those of the previous environments. Therefore, it is conceivable that these bacteria are equipped with mechanisms that allow them to cope with changing concentrations of sodium, a major solute of seawater.

These bacteria have two circular chromosomes, large (chromosome 1) and small (chromosome 2), both of which are indispensable for viability (11). Whereas chromosome 1 carries many essential genes involved in protein synthesis and housekeeping, chromosome 2 contains a number of genes encoding transporters and transcription regulators involved in environmental adaptation (12). The genomic information reveals that *Vibrio* species possess a single copy of the *secA*, *secY*, *secE*, and *secG* genes on chromosome 1. Because each of the SecA (13), SecY (14), and SecE (15) homologs of *Vibrio* can function when introduced into *E. coli* cells, the fundamental makeup of the Sec machinery may be common among these bacterial species. However, protein translocation into the *Vibrio* membrane vesicles in vitro was enhanced by the Na^+-motive force, instead of the proton-motive force (16). Furthermore, protein export in *E. coli* cells expressing the *V. alginolyticus* SecDF1 (V.SecDF1) protein (discussed below) was enhanced by Na^+ (5). Whereas the sodium dependence of the *Vibrio* SecDF is in accord with their marine origin, *V. alginolyticus* and other *Vibrio* species actually have two sets of *secDF* genes, which we designate *secDF1* and *secDF2*, on chromosomes 1 and 2, respectively. The present study addresses whether these SecDF homologs play different roles in the bacterial physiology and how their expression is regulated.

A remarkable mechanism is known about regulation of protein targeting/translocation machineries, in which specific monitoring substrates directly monitor the activities of the respective systems; SecM monitors the Sec protein export activity in *E. coli* (17, 18)

Significance

Bacteria living in seawater must cope with low-sodium environments that they may encounter. Here we show an unexpected finding that remodeling of the Sec protein export machinery plays a pivotal role in this adaptation. *Vibrio alginolyticus* possesses alternative SecDF1 and SecDF2 homologs that use the transmembrane gradient of Na^+ and that of H^+, respectively, to enhance protein export by cooperating with the SecYEG translocon. The synthesis of SecDF2 is induced in low-Na^+ environments, and this induction is essential for the bacterium to survive low salinity. Remarkably, the *Vibrio* species use a nascent polypeptide, VemP, to monitor the functional state of the Sec pathway and to up-regulate translation of SecDF2 when activity of the SecDF1-containing Sec machinery declines.

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and MifM monitors the YidC protein insertion activity in *Bacillus subtilis* (19). They up-regulate translation of *secA* and *yidC2*, respectively, in response to defects in the respective protein localization pathways, thereby contributing to the homeostasis of cellular protein localization processes (20–23). SecM and MifM, called regulatory nascent polypeptides, undergo translation arrest in a regulated manner such that the arrest is released upon engagement of their nascent chains in the localization processes (17–19, 24–26). The arrested ribosome disrupts the secondary structure of the mRNA to expose the ribosome-binding (Shine–Dalgarno) sequence of the target gene, thus allowing the free ribosomes of the cell to initiate translation of the target gene. Here, we report that the *Vibrio* species of bacteria use a regulatory nascent polypeptide, VemP, to up-regulate translation of *secDF2* upon encountering a low-Na^+ environment, in which V.SecDF1 cannot function. Our results reveal that translation arrest contributes as an essential mechanism to the environmental adaptation in marine bacteria.

Results

***V. alginolyticus* Possesses Na^+-Dependent and Na^+-Independent SecDF Paralogs.** Although SecD1, SecF1, SecD2, and SecF2 are encoded by separate cistrons in *Vibrio*, we call the SecD-SecF complexes collectively V.SecDF1 and V.SecDF2 and their genes *secDF1* (*secD1-secF1* on chromosome 1) and *secDF2* (*secD2-secF2* on chromosome 2), respectively. In *V. alginolyticus*, $secDF1_{VA}$ is more closely related than $secDF2_{VA}$ to the *E. coli secD-secF* genes in the amino acid sequences of the encoded proteins (Fig. S1) as well as in the *yajC-*$secD1_{VA}$*-*$secF1_{VA}$ arrangement, presumably forming an operon (Fig. 1*A*). The $secD2_{VA}$ *and* $secF2_{VA}$ genes on chromosome 2 are preceded by an ORF (termed *vemP* in this work, as shown below), which is conserved among *Vibrio* species but unrelated to *yajC*. To elucidate functions of these V.SecDF paralogs, we expressed them in *E. coli* cells carrying the *secD1* (Cs) mutation that virtually eliminated the endogenous SecDF expression and impaired protein export severely (Fig. 1*B*, lane 9). When V.SecDF1 was expressed, export of outer membrane protein A (OmpA) and maltose-binding protein (MBP) was recovered markedly provided that the medium was supplemented with 300 mM NaCl (Fig. 1*B*, compare lanes 1 and 3) but not KCl (lane 2) (5). Such Na^+ dependence was not observed when the mutant cells were complemented with V.SecDF2 or *E. coli* SecDF, which fully corrected the defect even without added NaCl (Fig. 1*B*, lanes 4–7). These results suggest that V.SecDF1 is unable to use the proton gradient and can instead use the Na^+ gradient to execute its protein export function, whereas V.SecDF2 is independent of Na^+ and presumably H^+-dependent, as has been shown for the *E. coli* SecDF protein (5). Thus, V.SecDF1 has been optimized to the primary habitat, seawater, of the *Vibrio* strain whereas V.SecDF2 has retained or gained (27) the proton dependence.

SecDF2 Is Inducible upon Na^+ Deprivation and Crucial for the Life of *V. alginolyticus* in Low-Na^+ Environments. To examine Na^+ dependence of *V. alginolyticus* growth, we used M63-amino acids medium supplemented with a total of 300 mM of NaCl–KCl combinations. A $secDF1_{VA}{}^+$ $secDF2_{VA}{}^+$ strain VIO5 (28), called WT strain hereafter, was able to grow in the presence of as low as 13 mM of Na^+, albeit at a slightly reduced rate than in the presence of higher concentrations of Na^+ (Fig. 2*A*, *Left* and Fig. S2*C*). Thus, this halophilic *V. alginolyticus* strain can adapt to limited-Na^+ environments as long as the total ionic strength of the medium is maintained. We constructed *V. alginolyticus* mutants deleted for $secDF1_{VA}$ (Δ*secDF1*) or $secDF2_{VA}$ (Δ*secDF2*) (*SI Materials and Methods* and Fig. S2*D*). The Δ*secDF1* mutant exhibited Na^+-independent growth similarly to the WT strain (Fig. 2*A*, *Middle*). By contrast, the Δ*secDF2* mutant depended heavily on the Na^+ concentrations; it showed maximum growth rate in the presence of 300 mM Na^+, which was progressively declined at lower Na^+ concentrations

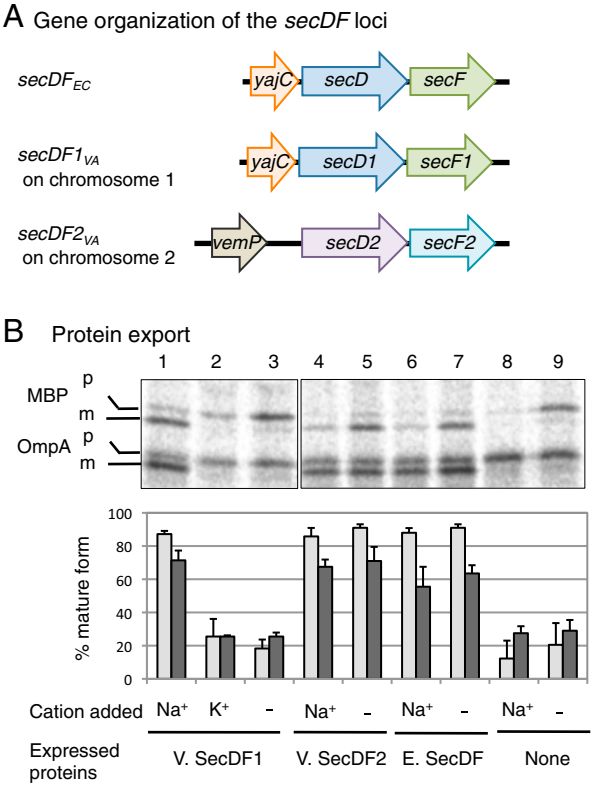

Fig. 1. Cation dependence of the V.SecDF paralogs expressed in *E. coli*: V.SecDF1 requires Na^+. (*A*) Gene organization of the *secDF* loci in *E. coli* and *V. alginolyticus*. ORFs are shown by arrows. (*B*) Protein export-enhancing activities of V.SecDF1 and V.SecDF2 assessed using the *E. coli secD1*(Cs) mutant. *E. coli secD1* cells expressing the indicated SecDF protein were grown in M63 medium supplemented with 300 mM Na^+ or K^+, as indicated, at 37 °C until an early log phase, shifted to 20 °C, and cultivated for additional 30 min. Cells were pulse-labeled with [^{35}S]methionine for 1 min. Immunoprecipitated precursor (p) and mature (m) forms of labeled MBP and OmpA were separated by 10% Laemmli gel and detected by phosphorimaging (*Upper*). Export efficiencies (proportions of mature form) of MBP (gray) and OmpA (black) are shown with SD ($n \geq 3$) (*Lower*). See also Fig. S1.

(Fig. 2*A*, *Right*). Thus, V.SecDF2 is essential for growth in the absence of sufficient levels of Na^+.

The differential roles played by the two paralogs of SecDF were corroborated by immunologically detecting them (Fig. S2 *A* and *B*). V.SecD1, detected as a doublet band, persisted in the WT *V. alginolyticus* strain grown at any concentrations of Na^+, with a slight decrease at lower Na^+ concentrations (Fig. 2*B*, *Upper*, lanes 1–4). By contrast, V.SecD2 was undetectable in the presence of 300 mM Na^+ (Fig. 2*B*, *Lower*, lane 4 and Fig. S2*C*, *Lower*, lanes 6–10). It became detectable at 63 mM or lower concentrations of Na^+, in an inversely concentration-dependent manner (Fig. 2*B*, *Lower*, lanes 1–3 and Fig. S2*C*, *Lower*, lanes 1–5). We eliminated the possibility that the apparent induction of V.SecD2 at low Na^+ concentrations was due to its selective degradation at higher Na^+ concentrations by pulse-labeling and immunoprecipitation experiments. The synthesis rate of V.SecD2, assessed by brief pulse-labeling of *V. alginolyticus* cells with [^{35}S]methionine, was indeed repressed at 300 mM Na^+ (Fig. 3*A*, lane 1) and induced at lower Na^+ concentrations (Fig. 3*A*, lanes 2–5). We also found that the synthesis of V.SecF2 was regulated almost identically to V.SecD2 (Fig. S3*A*, *Right*, lanes 1 and 4).

We assessed protein export abilities of the *V. alginolyticus* strains by pulse-labeling MBP. Rapid export of MBP was observed with WT and the Δ*secDF1* cells, as shown by preferential labeling of the mature form, irrespective of the Na^+ concentrations (Fig. 2*C*,

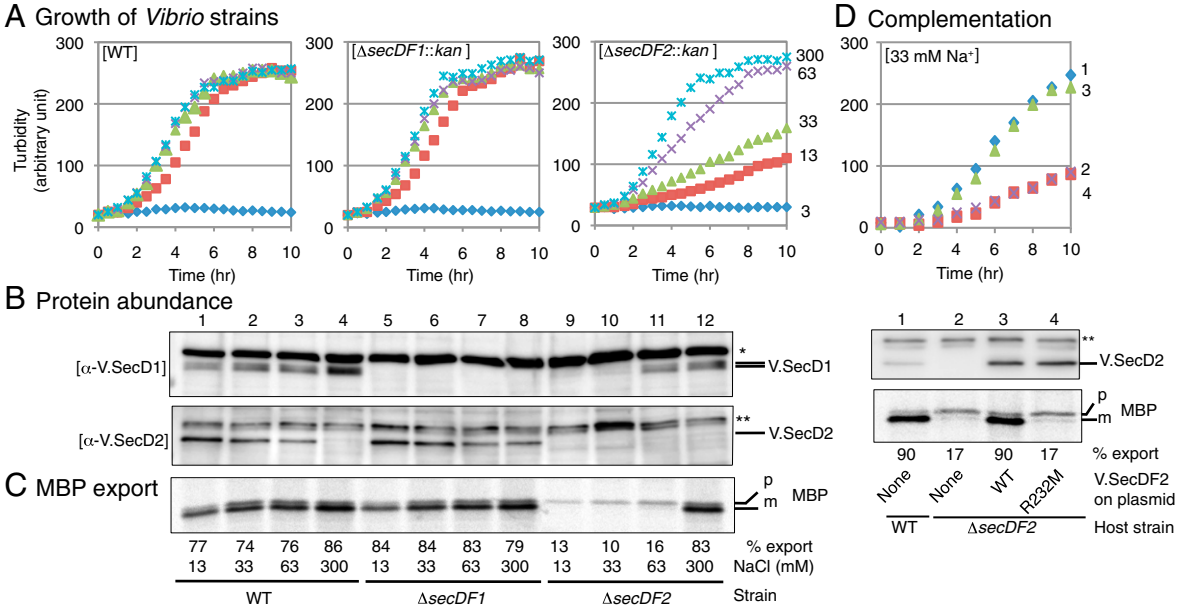

Fig. 2. Physiological roles of V.SecDF1 and V.SecDF2 in *V. alginolyticus*. (*A*) V.SecDF2 is important for growth under Na^+-limited conditions. WT and the indicated deletion mutants of *V. alginolyticus* were grown at 30 °C in M63 medium supplemented with a total of 300 mM of NaCl and KCl, in which Na^+ concentrations were 3 (blue), 13 (brown), 33 (pale green), 63 (purple), and 300 (pale blue) mM. Cell densities were monitored by turbidity measurements. (*B*) Cellular abundance of V.SecD2 correlates inversely with Na^+ concentrations and the presence of V.SecDF1. *V. alginlyticus* strains indicated at the bottom of *C* were grown at 30 °C until a mid log phase in the media supplemented with the indicated concentrations of Na^+. Total cellular proteins of equivalent amounts were separated by Laemmli SDS/PAGE, followed by immunodetection of V.SecD1 (*Upper*) and V.SecD2 (*Lower*). Asterisks show nonspecific background bands. (*C*) Severe protein export defects in the absence of V.SecDF2 and sufficient Na^+ levels. The same *Vibrio* strains used in *B* were grown at 30 °C in M63 medium supplemented with the indicated concentrations of Na^+ and pulse-labeled for 30 s. Extracts of equivalent radioactivities were used for immunoprecipitation of MBP. Upon electrophoresis, precursor (p) and mature (m) bands of MBP were quantified to give the efficiencies of processing (export) shown at the bottom. (*D*) Activities of V.SecDF2 to support growth and protein export. (*Upper*) Growth at 30 °C in M63 medium supplemented with 33 mM NaCl and 10 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) was followed for the following *V. alginolyticus* strains: 1, WT (VIO5) carrying empty vector and 2–4, Δ*secDF2* carrying the vector (2), carrying the V.SecDF2-expressing plasmid (3), and carrying the V.SecDF2(R232M)-expressing plasmid (4). (*Middle*) Cellular abundance of V.SecD2 was examined by anti-V.SecD2 immunoblotting. Asterisks show nonspecific background signals. (*Lower*) Export of MBP was followed for the above strains by [^{35}S]methionine pulse-labeling for 1 min at 30 °C. Processing efficiencies (percent) are shown at the bottom. See also Figs. S2 and S3.

lanes 1–8). By contrast, MBP export in the Δ*secDF2* cells was severely impaired, as judged from the exclusive labeling of its precursor form (Fig. 2*C*, lanes 9–11), unless cells were grown in the Na^+-rich (300 mM) medium (Fig. 2*C*, lane 12). The efficiencies of MBP export correlated well with the concentrations of Na^+ (Fig. S3*B*), although we observed that labeling of the MBP species was reduced under the export-compromised conditions for unknown reasons. The growth and export defects of the Na^+-limited Δ*secDF2* cells were rescued when functional V.SecDF2 was supplied in *trans* (Fig. 2*D* and Fig. S3*C*), excluding the possibility that the chromosomal deletion mutation affected expression of some other growth/export-related genes. These results establish that *Vibrio* cells adapt to sodium-limited environments by inducing V.SecDF2 capable of using the proton-motive force.

We noted that the cellular abundance of V.SecD1 and V.SecD2 exhibited some interdependence. First, V.SecD2 (Fig. 2*B*, lanes 5–8) and V.SecF2 (Fig. S3*A*, *Right*, lanes 2 and 5) were markedly induced in the Δ*secDF1* mutant cells, as if they compensated for the loss of V.SecDF1. Second, the cellular abundance of V.SecD1 decreased when Δ*secDF2* mutant cells were grown in the presence of 33 mM or lower concentrations of Na^+ (Fig. 2*B*, lanes 9 and 10).

***Vibrio* Cells Sense Lowered Functionality of the Sec Protein Export Pathway, Instead of Directly Sensing the Decreased Na^+ Concentrations, to Up-Regulate V.SecDF2 Production.** *Vibrio* cells did not seem to sense extracellular Na^+ concentrations for regulating $secD2_{VA}$, because V.SecDF2 was induced markedly in the Δ*secDF1* cells even in the Na^+-rich medium (Fig. 2*B*, lane 8). Pulse-labeling experiments confirmed that this induction took place at the level of the synthesis of V.SecD2 (Fig. 3*B*, *Lower*, lane 2). We then considered a possibility that the bacterium senses lowered functionality of the SecA-SecYEG-SecDF1 machinery. As a simple method to address this possibility, we examined effects of NaN_3, an inhibitor of SecA, on the synthesis rate of V.SecDF2 (Fig. 3*B*). We first confirmed that NaN_3 is a potent inhibitor of protein export in *V. alginolyticus* by its ability to inhibit signal peptide processing of pulse-labeled pre-MBP molecules (Fig. 3*C*). We then demonstrated that the synthesis of V.SecD2 was up-regulated markedly after addition of NaN_3 (Fig. 3*B*, *Lower*, lanes 4–7). The V.SecD1 synthesis was unaffected (Fig. 3*B*, *Upper*). Thus, an impairment of the Sec protein export process results in specific up-regulation of V.SecDF2 synthesis.

VemP, Encoded by the Upstream ORF of $secD2_{VA}$, Is a Secretory Protein That Undergoes Translational Elongation Arrest in Response to a Failure in Efficient Export. The upstream ORF of the $secD2_{VA}$ gene consists of 159 codons. The deduced amino acid sequence shows that its N-terminal region contains a segment characteristic of a signal sequence for protein export; it is enriched with hydrophobic amino acids, which are preceded by two positively charged amino acids. We also note that the C-terminal ~25-residue segment of the ORF is well conserved among *Vibrio* species (Table S1). We name this gene *vemP*, for *Vibrio* protein export monitoring polypeptide. Remarkably, VemP executes the function of monitoring the Sec activity and controlling the expression of $secDF2_{VA}$ by undergoing regulated translational elongation arrest, as discussed below.

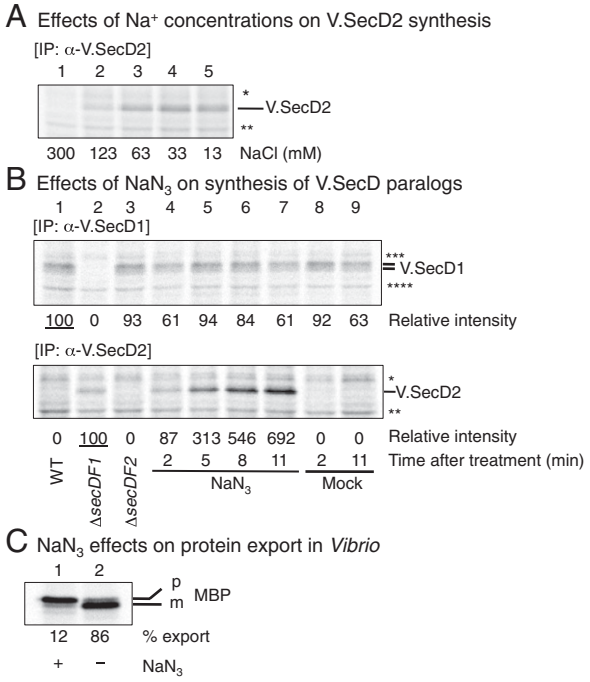

Fig. 3. The synthesis rate of V.SecD2 is affected not only by Na^+ but also by the absence of V.SecDF1 or the export-inhibiting azide. (*A*) WT *V. alginolyticus* grown in M63 medium supplemented with the indicated concentrations of Na^+ were pulse-labeled for 1 min with [^{35}S]methionine, followed by immunoprecipitation of V.SecD2. Asterisks indicate nonspecific background bands. (*B*) WT (lane 1 and lanes 4–9), Δ*secDF1* (lane 2), and Δ*secDF2* (lane 3) strains of *V. alginolyticus* grown in M63-300 mM Na^+ medium at 30 °C were pulse-labeled for 1 min without NaN_3 treatment (lanes 1, 2, 3, 8, and 9) or at the indicated time points after addition of NaN_3. Samples of equivalent radioactivities were subjected to immunoprecipitation with antibodies against V.SecD1 (*Upper*) and V.SecD2 (*Lower*). Asterisks indicate nonspecific background bands. The numbers at the bottoms show intensities of V.SecD1 relative to that in lane 1 (*Upper*) and those of V.SecD2 relative to that in lane 2 (*Lower*). (*C*) To verify that azide inhibits protein export in *V. alginolyticus*, cells were pulse-labeled for 0.5 min with (lane 1) or without (lane 2) 1 min NaN_3 pretreatment. Processing efficiencies of MBP are shown.

To characterize *vemP*, we expressed the plasmid-cloned *vemP-secDF2*$_{VA}$ genes in *E. coli*. Accumulation of the *vemP*-encoded protein was examined by immunoblotting. We used neutral pH SDS/PAGE to resolve protein species, which proved to include peptidyl-tRNA forms. Also, we focused on the effects of NaN_3, a Sec inhibitor, because VemP has the putative signal sequence for export across the membrane. Indeed, VemP was detected as different molecular forms in response to administration of NaN_3. Without NaN_3, a majority of VemP was electrophoresed at the position indicated as "m" (Fig. 4*A*, lane 1) expected for the signal sequence-processed molecular species, along with two faint bands, indicated as "p" (for precursor form) and "a" (for arrest product; discussed below) of slower migration. When cells were treated with NaN_3 for 30 min, the VemP species at the positions of "a" and "p" increased strikingly, whereas the "m" material diminished (Fig. 4*A*, lane 2). We reasoned that the large electrophoretic retardation of the "a" band was due to the presence of a covalently linked tRNA (26, 29); namely, translation elongation of VemP was arrested near the stop codon. In support of this possibility, treatment of the sample with RNase A converted the band "a" product to a material that migrated at the position "p" (Fig. 4*A*, lane 8). We note that VemP′-tRNA that gave band "a" was transferred poorly upon immunoblotting, explaining the intensity increase observed at position "p" after the RNase treatment (Fig. 4*A*, lanes 7 and 8). These results reveal two properties of VemP. First, it is indeed a secretory protein, which is handled by the SecA-SecYEG export pathway. Second, translation of VemP is subject to elongation arrest before termination, especially when its export is blocked. The materials that migrated at "p" may have represented either the polypeptide moiety of the arrested VemP′-tRNA produced by the ester bond hydrolysis in vivo or in vitro or, alternatively, translation-completed but signal peptide-unprocessed molecules.

The increased mobility of "m" in comparison with "p" is ascribable to the removal of the signal peptide, because the position of "m" was virtually identical to the position of the signal sequence-deleted construct of VemP (ΔSS) that was treated with RNase A (Fig. 4*A*, compare lanes 1 and 9/10). Strikingly, the results without RNase treatment indicate that the ΔSS variant of VemP was produced as the peptidyl-tRNA form that was electrophoresed at the position close to "a" whether or not the cells were treated with NaN_3 (Fig. 4*A*, lanes 3 and 4). Thus, the WT product "m" was produced upon release from the arrested state, in which restored elongation (for the last three amino acid residues of VemP) accompanied the export-coupled signal peptide cleavage (discussed below). By contrast, no arrest release took place for the ΔSS construct. Taken together with the NaN_3 effects observed with WT VemP, the constitutive elongation arrest seen with ΔSS VemP supports the notion that elongation arrest of VemP takes place strongly when VemP does not receive an effective export reaction.

To further characterize translation of VemP, we then used the in vitro translation system with the purified translation factors of *E. coli* and the ribosomes from either *E. coli* or *V. alginolyticus*, which behaved almost identically in translating *vemP* (Fig. S4 *B* and *D*). In the in vitro reactions, VemP was synthesized mainly as the peptidyl-tRNA form (Fig. 4*B*, lanes 2–4), which was converted by RNase treatment to the lower-molecular-weight product with an electrophoretic mobility similar to that of the in vivo product "p" (Fig. 4*B*, lane 9). Thus, in vitro translation of *vemP* is arrested near the C terminus. A greater proportion of the products was at the peptidyl-tRNA position at earlier time points than after prolonged reaction (Fig. 4*B*, compare lanes 2 and 4), suggesting that slow and spontaneous arrest release took place in vitro even in the absence of the Sec factors. Primer extension interference (toeprint) experiments verified that the ribosome indeed stalls at a specific position on the *vemP* mRNA in vitro, producing a specific length of reverse transcript (toeprint signal) (Fig. 4*C*, lane 1). Its alignment with the patterns of dideoxy sequencing using the same primer as well as with toeprint signals of control samples (Fig. S4*A*) indicates that translation of *vemP* is arrested as the P-site of the ribosome encounters the Gln156 codon (CAG) (Fig. 4*C*). Northern blot analysis of the translation product demonstrated that the arrested VemP'-tRNA carried a $tRNA^{Gln}$ moiety (Fig. S4*B*), indicating that it resided in the P-site. Thus, the VemP-translating ribosome fails in peptidyl transfer reaction from the $VemP_{1–156}$-$tRNA^{Gln}$ to the A-site Phe-$tRNA^{Phe}$.

Determinants of the VemP Elongation Arrest. A variant of VemP that is chain terminated by an amber mutation at the 132nd codon, termed VemPΔC hereafter, did not exhibit elongation arrest in vivo (Fig. 4*A*, lanes 5 and 6) or in vitro (Fig. 4*B*, lanes 6–8), indicating that the C-terminal 27 residues are required for the elongation arrest. We determined the C-terminal end of this requirement by a stop codon scanning. The in vitro elongation arrest withstood a stop-codon placement at the 157th position or its downstream (Fig. S4*C*, lanes 1–3) but not at position 156 or its upstream (Fig. S4*C*, lanes 4–7). Thus, "arrest sequence" of VemP ends at Gln156. Toeprint experiments showed that the stop codon at position 157 stalled the ribosome even though the full complement of release factors were included in the reaction (Fig. 4*C*, lane 8), indicating that the amino acid identity at position 157 is not essential for the ribosomal dysfunction.

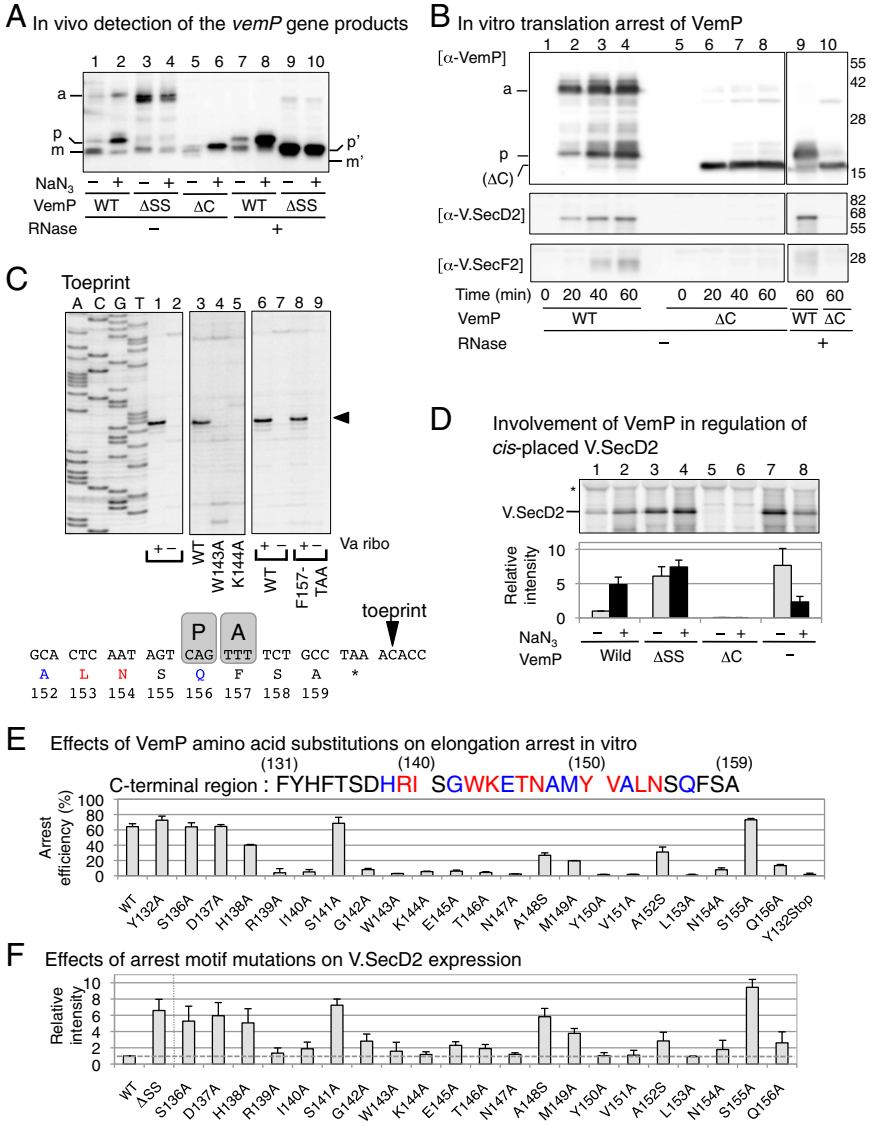

Fig. 4. Characterization of VemP as a Sec-monitoring substrate undergoing regulated translational arrest. (*A*) Detection of the *vemP* gene products. *E. coli* cells with plasmid carrying the *vemP-secDF2*$_{VA}$ genes containing the indicated *vemP* mutations were grown at 30 °C in M9-amino acids medium until a mid log phase. For the final 30 min of growth, they received 500 μM IPTG and 3 mM NaN_3 (lanes 2, 4, 6, 8, and 10). Total proteins of the cultures of equivalent amounts, with or without RNase A treatment (40 μg/mL for 30 min at 37 °C) as indicated, were separated by wide-range gel and detected with immunodetection of VemP. p′ and m′ indicate precursor and mature forms of VemP(ΔC), respectively. (*B*) In vitro translation of the *vemP-secD2*$_{VA}$*-F2*$_{VA}$ gene complex. DNA fragments of *vemP-secDF2*$_{VA}$ (WT) and *vemP(Y132amber)-secDF2*$_{VA}$ (ΔC) were used as a template for PURE System in vitro transcription–translation. At the indicated time points, samples, with or without RNase A treatment, were analyzed by neutral SDS/PAGE and immunoblotting detection of VemP (*Upper*), V.SecD2 (*Middle*), and V.SecF2 (*Lower*). (*C*) Ribosome stalling on the *vemP* mRNA studied by toeprinting. The *vemP* gene, with a mutation when indicated, was subjected to the PURE System reaction that lacked the *E. coli* ribosome (–) or that was supplemented with the ribosome prepared from *V. alginolyticus* (Va ribo) (+). The translation complexes were then used as a template for the reverse transcriptase reaction that was primed by a downstream primer (*SI Materials and Methods*). The cDNA products were electrophoretically separated along with the dideoxy sequencing ladders (left four lanes). Arrowhead indicates the signal generated by the ribosome stalling on *vemP*, which is schematically depicted below the toeprint gel on the basis of the comparison with the control reactions presented in Fig. S4*A*. P and A indicate the ribosomal P-site and A-site. (*D*) VemP controls *secDF2*$_{VA}$ expression in vivo. The plasmid-carried gene complex of *vemP-secD2*$_{VA}$*-F2*$_{VA}$ (lanes 1–6) with a *vemP* mutation (ΔSS, signal sequence deletion; ΔC, *Y132amber*) as indicated, as well as *secD2*$_{VA}$*-F2*$_{VA}$ DNA fragment directly placed under the control of the *lac* promoter on the vector (lanes 7 and 8) were induced in *E. coli* with 500 μM IPTG for 15 min at 30 °C. Cells were pulse-labeled with [^{35}S]methionine for 1 min with (+, black) or without (–, gray) 5 min NaN_3 (3 mM) pretreatment. Cell extracts of equivalent radioactivities were subjected to immunoprecipitation of V.SecD2. Intensities of the V.SecD2 band relative to that of the lane 1 sample are presented below the gel pattern ($n \geq 3$). (*E*) Effects of VemP amino acid substitutions on translation arrest in vitro. WT and mutant forms of *vemP* having the indicated amino acid substitution mutations were translated in vitro at 37 °C for 45 min in the presence of [^{35}S]methionine. Products were separated by neutral SDS/PAGE (Fig. S4*D*), and proportions (percent) of the arrested VemP-tRNA band in the sum of the tRNA-less VemP and the VemP-tRNA bands are presented ($n \geq 2$). Amino acid residues, whose mutations decreased the translation arrest to below 10% peptidyl-tRNA (red) and to 10–40% peptidyl-tRNA (blue) are highlighted at the top. (*F*) Effects of VemP amino acid substitutions on in vivo expression of V.SecD2. WT and mutant forms of Δ*ss-vemP-secD2*$_{VA}$*-F2*$_{VA}$ having the indicated amino acid substitution mutations were induced with 0.006% arabinose for 15 min in *E. coli* growing at 37 °C. Cells were then pulse-labeled for 1 min. Cell extracts of equivalent radioactivities were used for immunoprecipitation of V.SecD2, which was quantified after SDS/PAGE separation. Intensities of V.SecD2 relative to that of the WT sample are presented ($n \geq 3$). See also Figs. S4 and S5.

To further identify VemP elements required for the elongation arrest, we carried out alanine (serine when original residue was alanine) scanning mutagenesis of the C-terminal conserved segment. In vitro translation allowed us to determine the arrest-required amino acid residues, whose mutations are color-coded for strong (red) and moderate to weak (blue) defects in the arrest (Fig. 4*E* and Fig. S4*D*). Toeprint experiments confirmed defective ribosome stalling caused by the W143A and the K144A mutations (Fig. 4*C*, lanes 4 and 5). Thus, of the 21 amino acid residues of the segment examined, as many as 17 contribute to the translation halt of VemP. We also examined effects of the three mutations of the ribosomal protein L22 that were shown previously to compromise the SecM elongation arrest in *E. coli* (18) on elongation arrest of VemPΔSS expressed in *E. coli*. L22 (*rplV*) mutations, G91D and $\Delta M^{82}KR$, but not A93T, impaired the elongation arrest of VemPΔSS (Fig. S5*A*). Thus, the ribosome participates actively in the VemP-based regulation, but nascent chain–ribosome interactions are not identical for VemP and SecM, which differ totally in the primary sequences (see Fig. 7*B*). These results show that a specific amino acid sequence in the nascent VemP polypeptide is responsible for the elongation arrest through its interaction with the ribosomal exit tunnel.

VemP Regulates Translation of V.SecDF2 and Is Essential for the *Vibrio*'s Adaptation to Environments of Different Sodium Levels. To study roles of VemP in the synthesis of V.SecDF2, we expressed the *vemP-secDF2*$_{VA}$ gene complex in *E. coli*. Pulse-labeling experiments showed that the V.SecD2 synthesis was up-regulated markedly by NaN_3 treatment (Fig. 4*D*, lanes 1 and 2). The V.SecD2 synthesis-enhancing effect was not specific for the chemical NaN_3, because an independent, genetic means to impair the Sec function also led to the up-regulated synthesis of V.SecDF2 in an *E. coli secY* mutant (Fig. S5*C*). By contrast, a direct connection of *secDF2*$_{VA}$ to the *lac* promoter resulted in NaN_3-independent production of V.SecD2, which was rather lowered by the drug, presumably due to general inhibition of metabolism (Fig. 4*D*, lanes 7 and 8). Thus, VemP is required for the regulation. The amber mutation at the 132nd codon of *vemP,* which produced the arrest-defective VemPΔC, prevented the V.SecD2 synthesis even when cells were treated with NaN_3 (Fig. 4*D*, lanes 5 and 6). The VemP function to support the NaN_3-dependent up-regulation of V.SecDF2 synthesis was *cis*-specific, because coexpression of WT VemP singly from a compatible plasmid did not restore V.SecDF2 synthesis from *vemP(Y132amber)-secDF2*$_{VA}$ (Fig. S5*D*, compare lanes 2 and 4). In contrast to the arrest-defective mutation, deletion of the VemP signal sequence, which caused constitutive arrest, allowed high-level and NaN_3-independent synthesis of V.SecD2 (Fig. 4*D*, lanes 3 and 4). Good correlations were also observed between the extents of VemP arrest and the levels of V.SecDF2 expression among the VemP and the *rplV* (L22) missense mutations that affected the arrest efficiencies (Fig. 4, compare *E* and *F*, and Fig. S5*A*). The effects of the *vemP* and the *rplV* missense mutations rule out any polarity effects on the expression of the downstream target, *secDF2*$_{VA}$. We then recapitulated this regulation in vitro. In the in vitro coupled transcription–translation system, *vemP*, *secD2*$_{VA}$, and *secF2*$_{VA}$ were transcribed into a single mRNA (Fig. S5*B*), from which both V.SecD2 and V.SecF2 were translated (Fig. 4*B*, lower panels, lanes 2–4). However, arrest-defective VemPΔC did not allow the synthesis of V.SecD2 or V.SecF2 (Fig. 4*B*, *Lower*, lanes 6–8) without affecting the mRNA production. From these results, taken together, we conclude that the *cis* placement of *vemP* upstream of *secDF2*$_{VA}$ is required for the regulated translation of V.SecD2.

To address whether VemP plays crucial roles in the V.SecD2 production and consequent adaptation to low-sodium environments in *V. alginolyticus*, we introduced the elongation arrest-impairing Tyr132 amber mutation into the *vemP* gene on chromosome 2. Remarkably, the mutant *V. alginolyticus* cells did not contain any detectable amount of the V.SecD2 protein (Fig. 5*C*, lane 4). Their ability to export MBP was markedly impaired as well (Fig. 5*B*, lane 4). Furthermore, the mutant cells exhibited marked growth retardation in medium containing 33 mM Na^+ (Fig. 5*A*, *Right*, growth curve 4), but not in the presence of 300 mM Na^+ (Fig. 5*A*, *Left*). The observed defects in protein export and growth were similar to those observed with the Δ*secDF2* mutant (Fig. 5, strain 3) and, in fact, complemented by the forced expression of WT V.SecDF2 from a plasmid (Fig. S5*E*, lane 3). A nonfunctional SecDF2 variant was incompetent in this complementation (Fig. S5*E*, lane 4). These results establish that the VemP nascent polypeptide has an essential role in the adaptation of the marine *Vibrio* to low-sodium environments through its regulated elongation arrest that enables the efficient translation of the *secD2*$_{VA}$-*secF2*$_{VA}$ genes.

Finally, we address the importance of the elements in the intergenic regions of the *vemP-secD2-secF2*$_{VA}$ mRNA that support the VemP-based regulation. A secondary structure model by CentroidHomfold (www.ncrna.org/centroidhomfold/) shows that the ribosome-binding (Shine–Dalgarno) sequence of *secD2*$_{VA}$ is likely to be sequestered in a stem structure (Fig. 6*A*). The ribosome stalling at the arrest point in *vemP*, close to the stem-forming region, is then expected to interfere with the stem-loop formation, and, therefore, to liberate the Shine–Dalgarno sequence for initiation of the *secD2*$_{VA}$ translation by the free ribosomes. To experimentally validate the above model, we increased the distance between the arrest point and the secondary structure-forming region, by an insertion of one or two units of an 18-nucleotide sequence (Fig. 6*A*). Such a manipulation resulted in a severely impaired induction of the V.SecD2 synthesis upon export inhibition (Fig. 6*B*), without affecting the VemP translation arrest

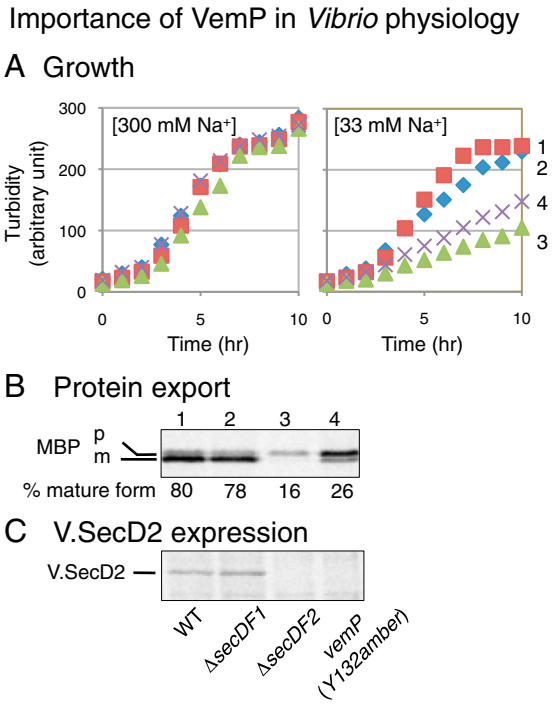

Fig. 5. VemP with the normal ability to arrest its own translation is essential for *V. alginolyticus* to grow rapidly and induce V.SecDF2. (*A*) Growth of *V. alginolyticus* strains with mutations as indicated by numbers (1, WT, squares; 2, Δ*secDF1,* diamonds; 3, Δ*secDF2,* triangles; 4, *Y132amber*, crosses) was followed at 30 °C in M63 minimum medium supplemented with either 300 mM Na^+ (*Left*) or 33 mM Na^+ (*Right*). (*B* and *C*) The *Vibrio* strains used in *A* were grown in the M63 medium supplemented with 33 mM Na^+ until an early log phase and then pulse-labeled with [^{35}S]methionine for 30 s. Labeled MBP and V.SecD2 were immunoprecipitated and separated by 10% SDS/PAGE, followed by phosphor imaging. See also Fig. S5.

per se (Fig. 6*C*). Thus, the regulatory significance of the ribosome stalling requires its occurrence in the close vicinity of the secondary structure-forming unit of the mRNA. The fact that V.SecF also receives this regulation may rest on the short $secD2_{VA}$-$secF2_{VA}$ intergenic region, having only two nucleotides (Fig. 6*D*). Here, the translational coupling mechanism (30, 31) may allow coordinated regulation of $secD2_{VA}$ and $secF2_{VA}$ by VemP.

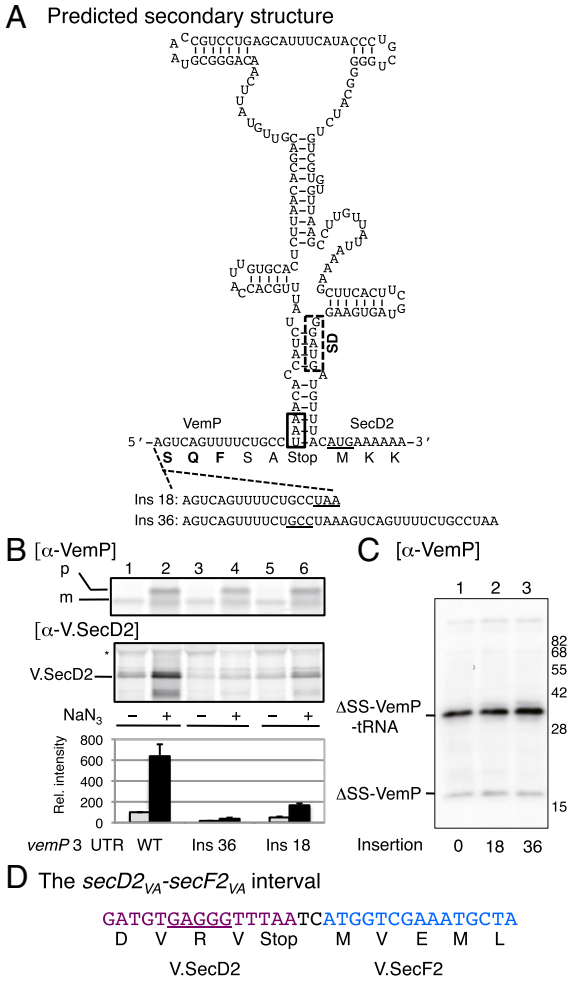

Fig. 6. Regulatory importance of the *vemP-secD2-secF2* operon arrangement. (*A*) Predicted secondary structure of mRNA at the *vemP-secD2*$_{VA}$ intergenic region. The RNA sequence from the fifth last codon of *vemP* to the third codon of *secD2*$_{VA}$ are shown with the secondary structure predicted by CentroidHomfold. The putative SD sequence and the start codon of *secD2*$_{VA}$ are indicated by box and underline, respectively. The P site and the A site of the VemP-stalled ribosome (Fig. 4*C*) are shown schematically. To separate the VemP arrest point and the secondary structure-forming region, we inserted one or two copies of the 18 nucleotides encoding the last five amino acid residues of VemP followed by the termination codon, shown at the bottom. (*B*) Separation of the arrest point and the stem-loop-forming region impairs the regulation. *E. coli* cells carrying indicated *vemP-secDF2*$_{VA}$ plasmids (with or without the insertion mutations shown in *A*) were induced with IPTG for 15 min at 37 °C and treated with (+) or without (–) 3 mM NaN_3 for 5 min. Cells were then pulse-labeled for 1 min. Cell extracts of equivalent radioactivities were subjected to anti-VemP (upper gel) and anti-V.SecD2 (lower gel) immunoprecipitation. In the upper gel, "p" indicates the signal peptide unprocessed form of VemP, which included the polypeptide part of the elongation arrested VemP, whereas "m" indicates the signal peptide-processed mature form. Relative intensities of V.SecD2 are shown in the bottom graph, taking the value of lane 1 as unity ($n = 3$). Gray and black bars represent results obtained without and with NaN_3 treatment, respectively. (*C*) The insertion mutations do not affect translation arrest of VemP. *E. coli* cells carrying pBAD24-Δ*ss-vemP-V.secDF2* with or without the insertion mutations were induced with 0.02% arabinose for 1 h at 37 °C. The same amounts of proteins were separated by 10% wide-range gel electrophoresis, followed by immunodetection of VemP. (*D*) The *secD2*$_{VA}$-*secF2*$_{VA}$ interval. The nucleotide sequence from the fourth last codon of *secD2*$_{VA}$ to the fifth codon of *secF2*$_{VA}$ is shown. The predicted SD sequence of *secF2*$_{VA}$ is underlined.

Discussion

SecDF uses the proton-motive force across the membrane to enhance protein export that has been initiated by the SecA-SecYEG core Sec components (5). It is important for the normal physiology of *E. coli*, because its depletion leads to severe defects in protein export and cell growth, especially at low temperatures (32). However, V.SecDF1 and V.SecDF2 are not simply redundant-essential factors in *Vibrio*s but differentiated with respect to the cations that energize the export-enhancing functions. In order for the *Vibrio* strain to cope with and survive the environments of changing salinity, they must use a proper V.SecDF paralog. We have presented pieces of evidence that support the above conclusions, including the following: (*i*) V.SecDF1 uses the sodium-motive force, whereas V.SecDF2 uses the proton-motive force; (*ii*) production of V.SecDF2 is inversely correlated with sodium concentrations of media; (*iii*) in this regulation, the Sec protein export activity is directly monitored by VemP, which undergoes regulated translation arrest to stimulate translation of V.SecDF2; and (*iv*) *V. alginolyticus* requires the VemP-mediated up-regulation of V.SecD2 production to grow and propagate rapidly under low-sodium conditions.

The constitutive expression of the sodium-dependent paralog, V.SecDF1, in *Vibrio* cells seems to be in accordance with the primary habitat, marine and estuarine water, of this species. The location of *secDF1*$_{VA}$ on the "housekeeping" chromosome 1 and the similarity of its organization to that of the *E. coli yajC-secD-secF* operon could suggest that the V.SecDF1 protein has evolved from the proton-using prototype during marine bacteria's adaptation to seawater (33). However, an alternative view that the sodium motive force was the primordial form of energy (27, 34) suggests that the proton-using SecDF homologs, including V.SecDF2, had evolved from a V.SecDF1-like ancestor. Whatever the evolutionary scenarios may be, the V.SecDF2 protein in *Vibrio*s today supports their life in such low-sodium environments as brackish water and intrabody or intracellular fluid of host animals. The regulated expression of SecDF2 should contribute to the prevalence of this group of bacteria in marine–estuarine interfaces.

The existence of the two V.SecDF paralogs with different ion selectivity is reminiscent of the dual flagella systems, Na^+- vs. H^+-driven, in *V. alginolyticus* (35). However, the H^+-dependent lateral flagella is induced in response to an increase in external viscosity (36). *V. alginolyticus* uses a distinct mechanism to maintain high protein export activity in different ionic environments by exchanging the SecDF paralogs (Fig. 7*A*). The secretory protein VemP possesses an "arrest sequence" that prevents the ribosome from continuing elongation beyond the Gln156 codon. The *secD2*$_{VA}$ translation remains switched on as long as the ribosome stalling continues, and the stalled ribosome liberates the Shine–Dalgarno sequence of the *secD2*$_{VA}$ gene (Fig. 6*A*). VemP, which may be called a "Sec-monitoring substrate," can give rapid, real-time on/off outputs for V.SecD2 translation in response to fluctuations of the cellular protein export activity. Moreover, this regulation is directly coupled to the translation of the other subunit, V.SecF2, of the complex through the translational coupling mechanism (Fig. 6*D*). We envisage that any other mechanisms that use a secondary signaling molecule, if any, will only be able to give greatly delayed outputs compared with the Sec-monitoring substrate, which can register the primary event in real time.

High-Na^+ environments (~600 mM in seawater) allow *Vibrio* to depend on the Na^+-driven V.SecDF1, which is abundantly present in the cell (Fig. 7*A*, *Left*). By contrast, low-Na^+ environments prevent V.SecDF1 from functioning, and the resulting decline in protein export is sensed by VemP, which immediately

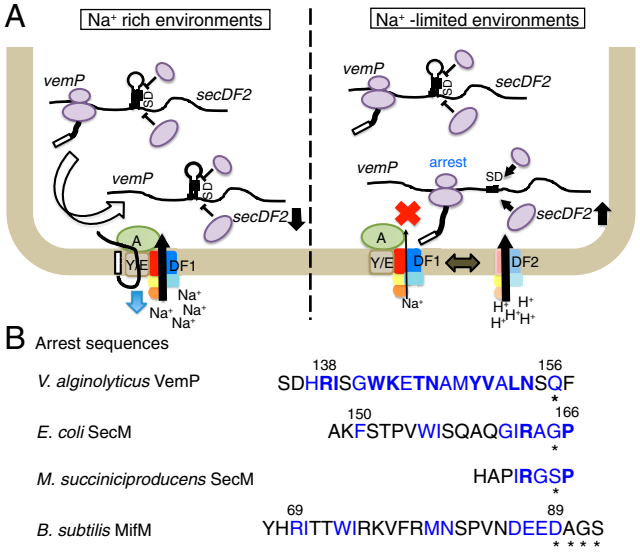

Fig. 7. (*A*) Adaptation of *Vibrio* species to salinity changes through export-controlled translation arrest of VemP. (*Left*) Repression of V.SecDF2 synthesis in Na^+-rich environments. Because the VemP export occurs efficiently by the V.SecDF1-containing Sec machinery capable of using the Na^+-motive force, the VemP elongation arrest is transient. In this situation, the duration of the arrest, in which the Shine–Dalgarno sequence of V.SecD2 is available, is short, leading to low-level synthesis of V.SecD2. (*Right*) Up-regulation of V.SecDF2 synthesis in Na^+-limited environments. In the absence of sufficient concentrations of Na^+, activity of the V.SecDF1-containing Sec machinery will decline, leading to retarded export and prolonged elongation arrest of VemP. This in turn results in higher rates of initiation of $secDF2_{VA}$ translation. Newly synthesized V.SecDF2 molecules will substitute for V.SecDF1 such that the bacteria can now use the proton-motive force to effectively localize their secretory proteins. (*B*) Diversity in the intrinsic class of arrest sequences. Shown are the amino acid sequences that induce translation arrest without any cofactor, which have been elucidated for VemP, SecM (18, 52), and MifM (19). Residues whose identities contribute to the elongation arrest are highlighted in blue; particularly important residues are shown in boldface. Asterisks indicate the last residue of the arrested peptidyl-tRNA, which resides in the P-site of the ribosome. See also Fig. S6.

activates translation of V.SecDF2 to enable cells to use the H^+ gradient for protein export. A recent transcriptome analysis of *V. parahaemolyticus* suggests that SecDF2 is a member of the low-salt stimulon (37). However, it remains to be determined whether the observed increase in the *secDF2* message under low-salt conditions was generated by transcriptional regulation or secondarily by the VemP-mediated translational regulation.

Importantly, the rapid up-regulation of the V.SecDF2 production will only be effective if the synthesized V.SecDF2 molecules can collaborate with the SecYEG translocon. Rapid subunit exchange and/or preferential translocon association of V.SecDF2 will help the immediate translocon remodeling. We suggest that a quick Sec machinery remodeling upon a cell's encounter with a low-salinity environment may be aided by preferential degradation of V.SecDF1 in the absence of sufficient levels of both V.SecDF2 and sodium ions (Fig. 2*B*). Decreased V.SecDF1 concentrations will then facilitate the formation of the SecDF2-containing export machinery that can use protons. Inversely, upon transition from a low-sodium to a high-sodium medium, the autogenously controlled V.SecDF2 synthesis will be terminated eventually due to cross-talks with now-activated V.SecDF1. In fact, the cellular abundance of V.SecD2 became negligible already at the time point of 3 h after the medium change (Fig. S2*C*), pointing to the possibility of active degradation. Possible involvement of a membrane protein quality control system (38) in *Vibrio*'s bidirectional adaptation to salinity changes will be an interesting research subject in the future.

Vibrio species lack the *secM* gene and instead have acquired *vemP*. It is intriguing that the Sec monitoring substrate in *E. coli* (SecM) and in *V. alginolyticus* (VemP) regulates the expression of different Sec components, SecA and SecDF, respectively. Probably, the *Vibrio* species chose V.SecDF2 as the target of regulation because SecDF2 carries out an environment-sensitive part of the export processes in this organism. The use of VemP seems to be advantageous because it can sense any conditions that disturb the protein export pathway to up-regulate V.SecDF2, which can use protons available ubiquitously. The rapid response mechanism by the monitoring substrate may be advantageous for *Vibrio* bacteria to infect animals successfully . A possible pathogenic importance of the V.SecDF2 paralog of *Vibrio* has been suggested by global transcriptome analyses, revealing *secDF2* induction upon infection (39, 40).

VemP adds to the concept, already discussed in the literature (19, 25, 41, 42), that each of the regulatory nascent polypeptides might have been acquired late in evolution by a specific group of species in phylogenetic lineages. Indeed, VemP is unique to *Vibrio* (Fig. S6*A*) and possibly other quite limited species. The high degree of conservation of the *vemP-secDF2* gene complexes (Fig. S6*A*) and the VemP arrest motifs (Table S1) as well as the stem-loop-forming potential of the *vemP-secDF2* intergenic regions (Fig. S6*B*) suggest that *Vibrio* species generally use VemP for regulation of *secDF2*, although the lengths of the intergenic regions are variable (Fig. S6*A*). In agreement with the late evolution theory, VemP has the arrest motif of unique amino acid sequence, which features as many as 17 amino acid residues that contribute to the temporary dysfunction of the translating ribosome. Because mutations of each one of these residues impair the arrest, the VemP elongation arrest must be a manifestation of their cooperative intramolecular interaction as well as interactions with ribosomal components. In line with the notion that ribosome arrest peptides interact with the ribosome in idiosyncratic ways (25, 43), ribosomal mutations affect the VemP-mediated arrest with a spectrum different from that observed for the SecM-mediated arrest. Furthermore, unlike the elongation arrest in SecM, which requires prolyl-tRNA in the A-site, that of VemP does not require a specific A-site amino acid; the P-site $VemP_{1–156}$-tRNA did not even receive efficient termination reaction when the A-site was programmed by a stop codon. Although the ribosome-tethered $VemP_{1–156}$-tRNA likely interferes with the ribosomal peptidyl transferase activity, the mode of its action is also different from that used by MifM, which induces multisite stalling of the *B. subtilis* ribosome, but not the *E. coli* ribosome (24, 44). We showed that VemP can stall the ribosomes from *V. alginolyticus* and *E. coli*, both being gram-negative, equally well. Species specificity of nascent polypeptide–ribosome interactions is an interesting subject to be explored.

It should be noted that VemP represents the third member of the "intrinsic" class of regulatory nascent polypeptides (45) that arrests translation without involving a small-molecular-weight cofactor (see refs. 46 and 47 for the latter examples). Instead, their translation-arrested states are subject to release by the nascent chain engagement in protein localization reactions (Fig. 7*B*) (18, 19, 25). The unique arrest sequence of VemP indicates that living organisms have evolved ribosome arrest sequences in the course of genetic information diversification. However, they must have exploited the regulatory potential of the arrest sequences by arranging them in a convergent way into a regulatory unit such as the $vemP\text{-}secD2_{VA}\text{-}secF2_{VA}$, the *secM-secA*, and the *mifM-yidC2* operons. The involvement of the large number of VemP amino acid residues in arrest will give a unique opportunity to study how the ribosome recognizes nascent polypeptides and how the dynamics of the nascent chain affects the translation progression (48). Whereas these molecular mechanisms deserve focused and combined approaches of biochemistry, genetics, and structural biology, we should also understand how living organisms integrate such mechanisms to survive changing environments.

Microorganisms in marine–estuarine interfaces may continue to provide unique insights into environmental adaptation.

Materials and Methods

Strains and Plasmids. Strains and plasmids used in this study are listed in Tables S2 and S3, respectively and details of the constructions are described in *SI Materials and Methods*.

SDS/PAGE. Proteins were separated by 10% Laemmli SDS/PAGE for analysis of MBP, OmpA, and V.SecD paralogs and by 10% neutral pH SDS/PAGE, either wide-range gel (Nacalai Tesque) or Nu-PAGE (Life Technologies), for analysis of VemP, VemP-tRNA, and V.SecF paralogs (26, 29).

Immunoblotting. Immunoblotting analysis was carried out according to the standard protocols described previously (49), using antisera against V.SecD1 (1/3,000), V.SecD2 (1/1,000), and VemP (after affinity-purified, 1/1,000), which were prepared as described in *SI Materials and Methods* and Table S4. Antisera against V.SecF1 and V.SecF2 were diluted 10^3-fold with PBS containing 5% (wt/vol) skim milk and 0.1% Tween 20 and pretreated with crude lysates prepared from the *Vibrio* Δ*secDF1* and Δ*secDF2* mutant strains, respectively, at 4 °C for 1 h to reduce background signals.

Pulse-Labeling Experiments. *E. coli* and *Vibrio* were cultivated in the specified minimal medium (*SI Materials and Methods*, *Media*) until an early log phase at 37 °C or 30 °C, respectively, and pulse-labeled with [^{35}S]methionine (ARS 0104A, 37 TBq/mmol) for 30 s or 1 min. The labeled cultures were immediately precipitated with ice cold TCA [final 5% (vol/vol)]. Immunoprecipitation was performed as described previously (50).

Purification of the *V. alginolyticus* Ribosomes. Purification of *Vibrio* ribosome was carried out according to the procedures described by Ohashi et al. (51) with modifications presented in *SI Materials and Methods*.

In Vitro Translation. In vitro transcription/translation reaction was carried out using PURE*frex* System (Gene Frontier Co., Ltd) in combination with either the *Vibrio* or the *E. coli* ribosomes, according to the manufacturer's instructions, as detailed in *SI Materials and Methods*.

Toeprint and Northern Blotting Dissection of the Ribosome-Nascent Chain Complexes on the *vemP* mRNA. The ribosomes from the *Vibrio* strain were combined with PURE*frex* System for in vitro translation/translation of the *vemP* gene. DNA templates for transcription/translation reactions were prepared by two successive PCR reactions as detailed in *SI Materials and Methods*. In vitro synthesized polypeptidyl-tRNAs of VemP derivatives were analyzed by Northern blotting using biotinylated oligodeoxynucleotide probes complementary to either $tRNA^{Gln}$ or $tRNA^{Phe}$ as described previously (24). Translation complexes were also subjected to toeprint analysis as described in *SI Materials and Methods*.

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1. Economou A, Wickner W (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell* 78(5):835–843.
2. Van den Berg B, et al. (2004) X-ray structure of a protein-conducting channel. *Nature* 427(6969):36–44.
3. Nouwen N, Piwowarek M, Berrelkamp G, Driessen AJ (2005) The large first periplasmic loop of SecD and SecF plays an important role in SecDF functioning. *J Bacteriol* 187(16):5857–5860.
4. Pogliano KJ, Beckwith J (1994) Genetic and molecular characterization of the Escherichia coli secD operon and its products. *J Bacteriol* 176(3):804–814.
5. Tsukazaki T, et al. (2011) Structure and function of a membrane component SecDF that enhances protein export. *Nature* 474(7350):235–238.
6. Mio K, et al. (2014) Conformational variation of the translocon enhancing chaperone SecDF. *J Struct Funct Genomics* 15(3):107–115.
7. Reilly GD, Reilly CA, Smith EG, Baker-Austin C (2011) Vibrio alginolyticus-associated wound infection acquired in British waters, Guernsey, July 2011. *Euro surveill* 16(42):19994.
8. Zhang L, Orth K (2013) Virulence determinants for Vibrio parahaemolyticus infection. *Curr Opin Microbiol* 16(1):70–77.
9. Noguchi T, et al. (1987) Vibrio alginolyticus, a tetrodotoxin-producing bacterium, in the intestines of the fish Fugu vermicularis vermicularis. *Mar Biol* 94(4):625–630.
10. Zhang L, et al. (2012) Type III effector VopC mediates invasion for Vibrio species. *Cell Reports* 1(5):453–460.
11. Makino K, et al. (2003) Genome sequence of Vibrio parahaemolyticus: A pathogenic mechanism distinct from that of V. cholerae. *Lancet* 361(9359):743–749.
12. Heidelberg JF, et al. (2000) DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. *Nature* 406(6795):477–483.
13. Kunioka E, Matsuyama S, Tokuda H (1998) Cloning and expression of the secA gene of a marine bacterium, Vibrio alginolyticus, and analysis of its function in Escherichia coli. *Gene* 216(2):303–309.
14. Bhattacharyya D, Das J (1997) The secY gene of V. cholerae: Identification, cloning and characterization. *Gene* 196(1-2):261–266.
15. Nishiyama K, Furuta M, Tokuda H (1998) Molecular cloning and functional characterization of SecE of a marine bacterium, Vibrio alginolyticus. *Biochem Biophys Res Commun* 251(3):894–897.
16. Tokuda H, Kim YJ, Mizushima S (1990) In vitro protein translocation into inverted membrane vesicles prepared from Vibrio alginolyticus is stimulated by the electrochemical potential of Na+ in the presence of Escherichia coli SecA. *FEBS Lett* 264(1):10–12.
17. Nakatogawa H, Ito K (2001) Secretion monitor, SecM, undergoes self-translation arrest in the cytosol. *Mol Cell* 7(1):185–192.
18. Nakatogawa H, Ito K (2002) The ribosomal exit tunnel functions as a discriminating gate. *Cell* 108(5):629–636.
19. Chiba S, Lamsa A, Pogliano K (2009) A ribosome-nascent chain sensor of membrane protein biogenesis in Bacillus subtilis. *EMBO J* 28(22):3461–3475.
20. Chiba S, Ito K (2015) MifM monitors total YidC activities of Bacillus subtilis, including that of YidC2, the target of regulation. *J Bacteriol* 197(1):99–107.
21. Murakami A, Nakatogawa H, Ito K (2004) Translation arrest of SecM is essential for the basal and regulated expression of SecA. *Proc Natl Acad Sci USA* 101(33):12330–12335.
22. Oliver DB, Beckwith J (1982) Regulation of a membrane component required for protein secretion in Escherichia coli. *Cell* 30(1):311–319.
23. Pogliano KJ, Beckwith J (1993) The Cs sec mutants of Escherichia coli reflect the cold sensitivity of protein export itself. *Genetics* 133(4):763–773.
24. Chiba S, Ito K (2012) Multisite ribosomal stalling: a unique mode of regulatory nascent chain action revealed for MifM. *Mol Cell* 47(6):863–872.
25. Ito K, Chiba S (2013) Arrest peptides: cis-acting modulators of translation. *Annu Rev Biochem* 82:171–202.
26. Muto H, Nakatogawa H, Ito K (2006) Genetically encoded but nonpolypeptide prolyl-tRNA functions in the A site for SecM-mediated ribosomal stall. *Mol Cell* 22(4):545–552.
27. Mulkidjanian AY, Dibrov P, Galperin MY (2008) The past and present of sodium energetics: May the sodium-motive force be with you. *Biochim Biophys Acta* 1777(7-8):985–992.
28. Okunishi I, Kawagishi I, Homma M (1996) Cloning and characterization of motY, a gene coding for a component of the sodium-driven flagellar motor in Vibrio alginolyticus. *J Bacteriol* 178(8):2409–2415.
29. Ito K, et al. (2011) Nascentome analysis uncovers futile protein synthesis in Escherichia coli. *PLoS One* 6(12):e28413.
30. Schoner BE, Belagaje RM, Schoner RG (1986) Translation of a synthetic two-cistron mRNA in Escherichia coli. *Proc Natl Acad Sci USA* 83(22):8506–8510.
31. Spanjaard RA, van Duin J (1989) Translational reinitiation in the presence and absence of a Shine and Dalgarno sequence. *Nucleic Acids Res* 17(14):5501–5507.
32. Pogliano JA, Beckwith J (1994) SecD and SecF facilitate protein export in Escherichia coli. *EMBO J* 13(3):554–561.
33. Deamer DW (1997) The first living systems: A bioenergetic perspective. *Microbiol Mol Biol Rev* 61(2):239–261.
34. Takekawa N, et al. (2015) Sodium-driven energy conversion for flagellar rotation of the earliest divergent hyperthermophilic bacterium. *Sci Rep* 5:12711.
35. Atsumi T, McCarter L, Imae Y (1992) Polar and lateral flagellar motors of marine Vibrio are driven by different ion-motive forces. *Nature* 355(6356):182–184.
36. McCarter L, Silverman M (1990) Surface-induced swarmer cell differentiation of Vibrio parahaemolyticus. *Mol Microbiol* 4(7):1057–1062.
37. Yang L, et al. (2010) The low-salt stimulon in Vibrio parahaemolyticus. *Int J Food Microbiol* 137(1):49–54.
38. Ito K, Akiyama Y (2005) Cellular functions, mechanism of action, and regulation of FtsH protease. *Annu Rev Microbiol* 59:211–231.
39. Xu Q, Dziejman M, Mekalanos JJ (2003) Determination of the transcriptome of Vibrio cholerae during intraintestinal growth and midexponential phase in vitro. *Proc Natl Acad Sci USA* 100(3):1286–1291.
40. Bisharat N, Bronstein M, Korner M, Schnitzer T, Koton Y (2013) Transcriptome profiling analysis of Vibrio vulnificus during human infection. *Microbiology* 159(Pt 9):1878–1887.
41. Cruz-Vera LR, Yanofsky C (2008) Conserved residues Asp16 and Pro24 of TnaC-tRNAPro participate in tryptophan induction of Tna operon expression. *J Bacteriol* 190(14):4791–4797.
42. van der Sluis EO, Driessen AJ (2006) Stepwise evolution of the Sec machinery in Proteobacteria. *Trends Microbiol* 14(3):105–108.
43. Wilson DN, Beckmann R (2011) The ribosomal tunnel as a functional environment for nascent polypeptide folding and translational stalling. *Curr Opin Struct Biol* 21(2):274–282.
44. Sohmen D, et al. (2015) Structure of the Bacillus subtilis 70S ribosome reveals the basis for species-specific stalling. *Nat Commun* 6:6941.

45. Ito K, Chiba S, Pogliano K (2010) Divergent stalling sequences sense and control cellular physiology. *Biochem Biophys Res Commun* 393(1):1–5.
46. Gong F, Ito K, Nakamura Y, Yanofsky C (2001) The mechanism of tryptophan induction of tryptophanase operon expression: tryptophan inhibits release factor-mediated cleavage of TnaC-peptidyl-tRNA(Pro). *Proc Natl Acad Sci USA* 98(16):8997–9001.
47. Ramu H, Mankin A, Vazquez-Laslop N (2009) Programmed drug-dependent ribosome stalling. *Mol Microbiol* 71(4):811–824.
48. Goldman DH, et al. (2015) Mechanical force releases nascent chain-mediated ribosome arrest in vitro and in vivo. *Science* 348(6233):457–460.
49. Shimoike T, et al. (1995) Product of a new gene, syd, functionally interacts with SecY when overproduced in Escherichia coli. *J Biol Chem* 270(10):5519–5526.
50. Baba T, et al. (1990) Characterization of cold-sensitive secY mutants of Escherichia coli. *J Bacteriol* 172(12):7005–7010.
51. Ohashi H, Shimizu Y, Ying BW, Ueda T (2007) Efficient protein selection based on ribosome display system with purified components. *Biochem Biophys Res Commun* 352(1):270–276.
52. Yap MN, Bernstein HD (2009) The plasticity of a translation arrest motif yields insights into nascent polypeptide recognition inside the ribosome tunnel. *Mol Cell* 34(2): 201–211.
53. Mori H, Akiyama Y, Ito K (2003) A SecE mutation that modulates SecY-SecE translocase assembly, identified as a specific suppressor of SecY defects. *J Bacteriol* 185(3): 948–956.
54. Terashima H, Koike M, Kojima S, Homma M (2010) The flagellar basal body-associated protein FlgT is essential for a novel ring structure in the sodium-driven Vibrio motor. *J Bacteriol* 192(21):5609–5615.
55. Miller JH (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
56. Kanehara K, Ito K, Akiyama Y (2002) YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA. *Genes Dev* 16(16): 2147–2155.
57. Val ME, Skovgaard O, Ducos-Galand M, Bland MJ, Mazel D (2012) Genome engineering in Vibrio cholerae: A feasible approach to address biological issues. *PLoS Genet* 8(1):e1002472.
58. Kusumoto A, et al. (2006) Regulation of polar flagellar number by the flhF and flhG genes in Vibrio alginolyticus. *J Biochem* 139(1):113–121.
59. Zhu S, Kumar A, Kojima S, Homma M (2015) FliL associates with the stator to support torque generation of the sodium-driven polar flagellar motor of Vibrio. *Mol Microbiol* 23, 10.1111/mmi.13103.
60. Le Roux F, Binesse J, Saulnier D, Mazel D (2007) Construction of a Vibrio splendidus mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. *Appl Environ Microbiol* 73(3):777–784.
61. Aiba H (1983) Autoregulation of the Escherichia coli crp gene: CRP is a transcriptional repressor for its own gene. *Cell* 32(1):141–149.
62. Silhavy TJ, Berman ML, Enquist LW (1984) *Experiments with Gene Fusions* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
63. Ito K, Akiyama Y (1991) In vivo analysis of integration of membrane proteins in Escherichia coli. *Mol Microbiol* 5(9):2243–2253.
64. Kihara A, Akiyama Y, Ito K (1995) FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. *Proc Natl Acad Sci USA* 92(10):4532–4536.
65. Akiyama Y, Ito K (1990) SecY protein, a membrane-embedded secretion factor of E. coli, is cleaved by the ompT protease in vitro. *Biochem Biophys Res Commun* 167(2): 711–715.
66. Mori H, Ito K (2006) The long alpha-helix of SecA is important for the ATPase coupling of translocation. *J Biol Chem* 281(47):36249–36256.
67. Nakatogawa H, Murakami A, Mori H, Ito K (2005) SecM facilitates translocase function of SecA by localizing its biosynthesis. *Genes Dev* 19(4):436–444.
68. Miller VL, Mekalanos JJ (1988) A novel suicide vector and its use in construction of insertion mutations: Osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. *J Bacteriol* 170(6):2575–2583.
69. Tokuda H, Nakamura T, Unemoto T (1981) Potassium ion is required for the generation of pH-dependent membrane potential and delta pH by the marine bacterium Vibrio alginolyticus. *Biochemistry* 20(14):4198–4203.
70. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177(14):4121–4130.
71. Morales VM, Bäckman A, Bagdasarian M (1991) A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* 97(1):39–47.