Enhanced arrest peptides

Exploration of arrest-peptide sequence space reveals arrest-enhanced variants*

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Background: The stalling efficiency of translational arrest peptides (APs) is sensitive to mechanical pulling forces on the nascent chain.

Results: We identify new APs with enhanced stalling efficiency.

Conclusions: Mechanical pulling forces reduce stalling induced by di-proline stretches in efp- cells.

Significance: Our results provide new insights into AP-induced translational stalling and offer new in vivo force sensors.

ABSTRACT

Translational arrest peptides (APs) are short stretches of polypeptide that induce translational stalling when synthesized on a ribosome. Mechanical pulling forces acting on the nascent chain can weaken or even abolish stalling. APs can therefore be used as in vivo force sensors, making it possible to measure the forces that act on a nascent chain during translation with single-residue resolution. It is also possible to score the relative strengths of APs by subjecting them to a given pulling force and ranking them according to stalling efficiency. Using the latter approach, we now report an extensive mutagenesis scan of a strong mutant variant of the Mannheimia succiniciproducens SecM AP, and identify mutations that further increase the stalling efficiency. Combining three such mutations, we designed an AP that withstands the strongest pulling force we are able to generate at present. We further show that di-proline stretches in a nascent protein act as very strong APs when translation is carried out in the absence of elongation-factor P (EF-P). Our findings highlight critical residues in APs, show that certain amino acid sequences induce very strong translational arrest, and provide a toolbox of APs of varying strengths that can be used for in vivo force measurements.

During protein synthesis, the nascent polypeptide chain moves through the approximately 100 Å long exit tunnel in the large ribosomal subunit (1). Strong interactions between the nascent chain and the tunnel might adversely affect protein synthesis and can even lead to complete blockage of the ribosome, a mechanism exploited by many antibiotics (2). It has therefore been postulated that the ribosome exit tunnel has a “teflon-like” surface that minimizes interactions with the nascent chain to avoid adverse effects on translation (1,3).

Nevertheless, some nascent chain segments are able to interact with the ribosomal exit tunnel in ways that block or slow down translation (4,5). In
bacteria, such translational arrest peptides (APs) are often used to regulate the translation of downstream open reading frames in polycistronic mRNAs (6,7). APs interact with distinct ribosomal RNA and protein components within the ribosomal exit tunnel (8), inducing conformations at the ribosome active site that can block the peptidyl-transfer reaction (9-11).

In the case of the AP-containing SecM protein in *Escherichia coli*, the arrest of nascent chain elongation can be overcome by the activity of the motor protein SecA, which presumably breaks the AP-tunnel interactions by mechanically pulling on the nascent chain (12). Based on this notion, we hypothesized that APs might find general use as transplantable *in vivo* force sensors, as indeed turned out to be the case (13-16).

The precise AP-tunnel interactions that lead to strong translational arrest are only partially known, and the arrest potency of related APs has not been systematically explored. We now report an extensive mutational screen of an unusually strong AP, a mutant variant of the SecM AP from *Mannheimia succiniciproducens* with the sequence HPPIRGSP, identifying substitutions that lead to substantial increases in resistance to pulling forces, and stronger translational arrest. By combining the most potent mutations, we have been able to construct APs that induce efficient ribosome stalling in *Escherichia coli* even under conditions of extremely strong pulling forces on the nascent chain.

In *E. coli* cells lacking elongation factor P (EF-P), di-proline stretches in nascent polypeptide chains can also cause ribosome stalling (3). To test whether this kind of stalling mechanism is sensitive to pulling forces, we have measured the strength of the translational arrest induced by di-proline stretches with different flanking residues in an *efp* strain, demonstrating their possible use as exceptionally short *in vivo* force sensors.

**EXPERIMENTAL PROCEDURES**

*DNA manipulations*—Site directed mutagenesis was performed using partial overlapping oligonucleotides (17) using previously generated constructs (13,16). Truncations were introduced by using fixed forward oligonucleotides and variable reverse oligonucleotides in a PCR reaction. Exchanges were performed by using the same partial overlapping oligonucleotide approach, but using both randomized positions in the forward oligonucleotides as well as specific sequences at the respective positions. Insertions were performed similarly, by using the inserted sequence as the partial overlapping sequence. The resulting product was *DpnI* treated and transformed into *E. coli* MC1061 cells. All products were transformed into MC1061 *E. coli* cells and confirmed by sequencing.

*Pulse-labeling*—*E. coli* MC1061, MG1655 and MG1655 *Δefp* cells, transformed with the respective constructs, were grown overnight at 37 °C in M9 minimal medium. The minimal medium contained 19 amino acids at a concentration of 1 µg ml⁻¹ but no methionine, 100 µg ml⁻¹ thiamine, 0.1 mM 0.4% (w/v) fructose and 100 µg ml⁻¹ ampicillin. The overnight cultures were back diluted 1:10 and grown for 3h to an OD₆₀₀ of 0.3. Protein expression was induced by the addition of 0.2% (w/v) arabinose for 5 minutes prior to the addition of [³⁵S]-methionine. After two minutes of pulse labeling, 0.5 sample volumes of ice cold 50% (v/v) trichloroacetic acid (TCA) was added to the sample and the samples were incubated for 30 minutes on ice. The samples were then centrifuged for 10 minutes at 20,800g at 4 °C and the pellet was washed with ice cold acetone, centrifuged again at 20,800g at 4 °C. The supernatant was removed and the pellet was resolubilized in 2% SDS Tris buffer (10mM Tris-Cl (pH 7.5), 2% (w/v) SDS) by vortexing and heating to 95 °C for 10 minutes. The sample was spun again to remove insoluble material and Lep protein was immunoprecipitated using a LepB polyclonal antibody. Samples were resolved on SDS-PAGE and visualized in a Fuji FLA-3000 phosphoimager. The full-length and the arrested form were quantified using Fujifilm Image Gauge and Easyquant software.

**RESULTS**

In order to screen for APs that can withstand strong pulling forces, we used a previously identified mutant version of the *M. succiniciproducens* SecM AP called SecM(Ms-Sup1) (13,16,18) as the starting template. The AP
was inserted close to the C-terminus of the *E. coli* inner membrane protein leader peptidase (LepB), Fig. 1a. LepB has two N-terminal transmembrane helices (TM1, TM2) and a large C-terminal periplasmic domain that is co-translationally translocated across the inner membrane by the SecYEG translocon. We used a modified LepB construct that contains a stretch of five aspartic acid residues (5D) in the periplasmic domain. The negatively charged 5D stretch is subjected to a strong pulling force generated by the electric membrane potential as it translocates across the inner membrane. The pulling force is detected as an increase in the fraction full-length protein (*f* _FL_ ) in constructs when the length _L_ of the linker that connects the 5D stretch to the P-site in the ribosome is 42–49 residues, Fig. 1b (16).

We chose a linker length _L_ = 46 residues that corresponds to a local minimum in the _f_ _FL_ profile (orange dot in Fig. 1b). The rationale behind this choice was that all minor changes in the location of the AP and linker region in the ribosomal tunnel and any attendant changes in the location of the 5D stretch relative to the membrane caused by mutations in the AP would result in an increase in _f_ _FL_ , and hence score as a weakening of the AP, whereas any increase in the arrest potency of the AP would lead to a decrease in _f_ _FL_. As shown in Fig. 1c, after immunoprecipitation of *in vivo* radiolabeled LepB[5D, _L_ = 46] we observe a full-length band (_FL_), a faster migrating arrested form of the protein (*A*), and an additional band due to ribosomal stacking behind the arrested ribosome (*). For this construct, _f_ _FL_ = 0.56. Any drop below this value would hence indicate a stronger AP.

**Mutagenesis of the SecM(Ms-Sup1) arrest peptide**

Using the LepB[5D, _L_ = 46] construct, we generated an extensive mutant library in which every residue of the eight-residue SecM(Ms-Sup1) AP HPPIRGSP, as well as two flanking residues on either side of the arrest peptide (SS and GS; these residues were introduced during cloning and are YF and QR in the wildtype SecM(Ms) AP), were changed to every other amino acid (see Materials & Methods). The 228 mutated constructs were transformed into *E. coli* MC1061 cells and _f_ _FL_ -values were determined in triplicate. All sequences and _f_ _FL_ -values are listed in the Supplementary Data (Tables S1 and S2), and the results are summarized in Fig. 2. The seven most C-terminal residues of the AP (residues -1 to -7) are all critical, and every substitution in this part of the AP, except for the S → (D, P) mutations, the P → (K, N, R) mutations, and the P → Y mutation resulted in very weak arrest (i.e., led to a strong increase in _f_ _FL_ ). Positions -8 to -10 showed a more graded response, with mutations to hydrophobic and, especially, tryptophan residues causing significant increases in the strength of the AP. Mutations in positions +1 and +2, immediately C-terminal to the AP, had no or little effect on _f_ _FL_.

Three mutations – S → P, S → W, and S → W – resulted in very strong arrest, with _f_ _FL_ reaching baseline levels (< 0.2). We introduced the two S → W mutations (plus the H → W mutation) individually and jointly into a construct with a stretch of 10 aspartic acid residues, LepB[10D, _L_ = 42], that generates a very strong pulling force (16). As seen in Fig. 3, _f_ _FL_ ≈ 1 in all three constructs with a single S → W exchange, i.e., these mutated APs can withstand the pulling force generated by 5 but not by 10 aspartic acid residues. Combining the three W mutations reduced _f_ _FL_ for the LepB[10D, _L_ = 42] construct to 0.2, indicating that their effects are additive.

To further characterize the S → W mutant, we obtained a full _f_ _FL_ profile for the LepB[10D, _L_ = 23–61] set of constructs, Fig. 4. Compared to the weaker SecM(Ms) and SecM(Ms-Sup1) APs, the SecM(Ms-Sup1;S → W) AP produces a much sharper main peak at _L_ = 41–42 residues, allowing us to determine the point of maximal pulling force for the 10D stretch with single-residue accuracy. On the other hand, the _f_ _FL_ values obtained with the S → W mutant are surprisingly high in the region _L_ = 23–36 residues, i.e. when the 10D stretch has not completely emerged from the ribosomal tunnel.

The S → P mutation appeared to dramatically increase the strength of the AP, since only arrested protein could be observed for the LepB[5D, _L_ = 46] construct ( _f_ _FL_ ≈ 0), Fig. 2. In this particular case, RNase-sensitive bands migrating slower than the full-length protein during SDS-PAGE were evident, Fig. 5a. These could either represent species with tRNA still bound to the nascent chain, or possibly covalently attached tmRNA added by the SsrA rescue system (19). When
introduced into the LepB[10D, L=42] construct, the S3→P mutation yielded \( f_{FL} \approx 0.3 \), Supplementary Fig. 5b, and no RNase-sensitive bands were observed. We further performed an alanine-scan of positions -1 and -3 to -8 of the SecM(Ms-Sup1;S3→P) AP in the LepB[10D, L=42] background. As expected, all these mutations led to a strong increase in \( f_{FL} \), Supplementary Fig. 5c, and no RNase-sensitive bands were apparent on the gels (data not shown). Thus, SecM(Ms-Sup1;S2→P) behaves like a strong AP under a force load, except that it has a more stably attached RNA than other APs.

As a final control we changed the codon for the serine residues in positions -9 and +2 to all six leucine codons in the LepB[5D, L=46] construct, Fig. 6. \( f_{FL} \) varied only within the margin of error between the different mutants, indicating that the observed changes in arrest-peptide strength depend on the introduced amino acid, rather than on the specific codon.

**AP function of oligo-proline stretches in an efp background**

In the absence of elongation factor EF-P, bacterial ribosomes stall efficiently already at two consecutive proline residues (3). It is not known, however, if stalling on oligo-proline stretches is sensitive to pulling forces on the nascent chain. To address this question, we used both a LepB construct that contains a stretch of 19 alanine residues at \( L = 39 \) residues, i.e., at a linker length that places the [19A] stretch in the translocon where it exerts only a weak pulling force on the nascent chain (13), and the LepB[10D, L=43] construct in which, as shown above, there is a strong pulling force, Fig. 7a. These constructs contained no internal oligo-proline stretches (the only naturally occurring PP doublet in LepB was replaced by AA).

As a control, we first tested the SecM(Ms) AP HAPIRGSP in the LepB[19A, L=39] construct in efp and efp' strains. In both cases, we observed a strong arrest, Fig. 7b. Upon replacement of the eight-residue long SecM(Ms) AP by eight glycine and serine residues (GSGSGGSS), only full-length protein could be observed in either the presence or absence of EF-P, as expected. Replacement of the C-terminal residue in the GSGSGGSS stretch by proline (lane “SG-1P”) led to no arrested protein in either the presence or absence of EF-P. However, the introduction of two or three proline residues (lanes “SG-2P” and “SG-3P”) led to robust stalling of LepB[19A, L=39] in the absence but not in the presence of EF-P.

In contrast, the SG-2P sequence only partially stalled the LepB[10D, L=43] construct in the absence of EF-P (\( f_{FL} = 0.5 \)), as seen in Fig. 7c. Stalling induced by oligo-proline stretches in the absence of EF-P thus can be at least partially overcome, but only by very strong pulling forces.

Given the strong arrest potency and the short length of the PP motif, we decided to test sequence variants of this motif by mutating the two flanking residues to every other amino acid and determining the strength of the individual variants in efp and efp' backgrounds. Varying residue \( X \) on the N-terminal side of a XPPZ motif (with \( Z = K \)) resulted in \( f_{FL} \)-values between 0.3 - 0.9 for the LepB[10D, L=43] construct, Fig. 8a and Supplementary Table S3, whereas the Z residue (with \( X = S \)) had only a minor effect on \( f_{FL} \), Fig. 8b. A similar analysis was recently published by Wilson and coworkers (20), using a β-galactosidase assay as a readout (i.e., the assay was carried out in the absence of a pulling force). The results for the X residue were similar in the two assays, Fig. 8c, except for \( X = A \) and \( X = P \) that both gave stronger arrest in the β-galactosidase assay than in the pulling force assay. In contrast to our results, however, Wilson et al. found a large variation in \( f_{FL} \) when the Z residue was varied, Fig. 8d. It thus appears that XPPZ-induced stalling has a different dependence on the Z residue in the absence or presence of a strong pulling force.

In order to test for the contribution of residues farther upstream of the PP motif, we mutated the four underlined residues in the preceding SGSGSGGS stretch to W, R, P, and D. Indeed, these mutations gave rise to a wide range of \( f_{FL} \) values, Fig. 9, in most cases conforming to the pattern seen for the SecM(Ms-Sup1) AP in Fig. 2.

Finally, we generated a full \( f_{FL} \) profile for the LepB[10D, L=23-52] series of constructs using PP-induced arrest in an efp’ background. As seen in Fig. 7d, the APPK stretch withstands even stronger pulling forces than does the SecM(Ms-Sup1;S9→W) AP, and the main peak is
sharper and has a clear maximum of $f_{RL} = 0.7$ at linker length $L = 42$ residues. Notably, the shoulder at $L \approx 23-36$ residues seen for the SecM(Ms-Sup1;S$_{0}$→W) AP is not present in this case, suggesting that it represents a specific effect elicited on the SecM(Ms-Sup1;S$_{0}$→W) AP by the highly negatively charged 10D-stretch.

**DISCUSSION**

SecM translational arrest peptides (APs) are sensitive to pulling forces acting on the nascent polypeptide chain (13-16). But just how much pulling force can they be made to withstand? Which residues in an AP are the most critical to the arrest potency, and hence interact most strongly with the ribosomal tunnel? Here, we show that the interactions between the SecM(Ms-Sup1) AP and the ribosomal exit tunnel can be tuned to considerably increase the strength of stalling. We also demonstrate that di-proline stretches, which induce stable translational stalling in EF-P deletion strains, effectively function as very short force-sensitive arrest peptides.

For the SecM(Ms-Sup1) AP, we substituted every amino acid for every other amino acid in the sequence SSHPPIRGSPGS (where the underlined segment is the AP as defined by Ala-scanning (18)) and analyzed the arrest potency under a rather strong pulling force. In positions -7 to -1, only two mutations were found to increase the strength of the AP, namely S$_{-2}$→P and P$_{-6}$→R (see Fig. 2). Mutations of residues S$_{-10}$, S$_{-9}$, and H$_{-8}$ to hydrophobic residues, and to W in particular, also markedly increased the arrest potency up to a point where the mutated AP could completely withstand the force generated by the electric potential across the inner membrane acting on a stretch of 5 but not of 10 consecutive aspartic acid residues (see Figs. 3 and 4). These positions are located in the vicinity of the constricted region of the ribosomal tunnel (8), and the effect of tryptophan in these positions may be similar to the tryptophan-binding site seen in the stalled TnaC AP, where bound L-tryptophan interacts with residue I$_{-10}$ in the AP (9). By simultaneously mutating the three positions -10 to -8 to tryptophan, we were able to generate an AP (WWPPIRGSP) that is able to stall translation even in the presence of the pulling force elicited by 10 aspartic acid residues, Fig. 3. This AP can, presumably, be made even stronger by introducing the S$_{-2}$→P and P$_{-6}$→R mutations, but, as the pulling force from a [10D] stretch is the strongest we have been able to generate thus far (16), we cannot determine if this is the case.

Two previous studies have reported AP motifs obtained from randomized sequence libraries using two related selection schemes (21,22). The most potent motifs found were GI(R/H)XPP, FxxYxIWPPP, R(S/A)PP, and HGPP. All four motifs are broadly consistent with the data reported in Fig. 2, and include the strong S$_{-2}$→P mutation. R$_{-4}$, which is absolutely critical in the SecM(Ms-Sup1) AP, is present in two of the four motifs, as is I$_{-5}$. Aromatic residues in position -7 (Y) and -10 (F) also have counterparts in the SecM(Ms-Sup1) AP mutational screen. A third study (18) identified revertants of a weakened variant of the *E. coli* SecM AP; again the results agree with ours (in particular, the strong stalling potency of a revertant AP with cysteine in position -10 was evident in this study, c.f., Fig. 2). Finally, we note that the 10 C-terminal residues in the *E. coli* SecM AP (SQAQGIRAGP) differ from the SecM(Ms-Sup1) AP by sequence changes that, according to the data in Fig. 2, should make it less potent, as indeed is the case (13,18).

The recent discovery that oligo-proline stretches stall translation in the absence of EF-P (3,20) prompted us to investigate whether this kind of stalling also could be overcome by pulling on the nascent chain. Similarly to the “classic” APs discussed above, we found that translational stalling at a di-proline stretch was relieved only by a very strong pulling force [10D], Fig. 7. Furthermore, we corroborate earlier evidence that the potency of the PP stalling motif is sensitive to the identity of the immediate N-terminal flanking residue (23,24), Fig. 8, and can thus be modulated to some extent.

In summary, we find that it is possible to design translational APs that are considerably more resistant to mechanical force than naturally occurring SecM APs, and that might even be expected to fully stall translation under almost any conceivable pulling force that can be generated in vivo. We further find that translational stalling induced by the most potent di-proline stretches in the absence of EF-P can be overcome, but only by
very strong pulling forces. The collections of SecM and di-proline APs reported in Fig. 2 and Fig. 8 considerably expand the toolbox of transplantable force sensors with different “spring constants” that can be used to measure mechanical forces generated on a nascent chain by various cotranslational processes, such as protein folding and membrane insertion/translocation.
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FIGURE LEGENDS

FIGURE 1. (a) Design of LepB[5D] constructs. A schematic picture of a translating ribosome bound to the SecYEG translocon is shown. AP: arrest peptide, L: number of residues in the linker between the [5D] stretch and the C-terminal proline residue at the end of the AP, F: pulling force generated by the membrane potential (ΔΨ) on the nascent chain. (b) Fraction of full-length protein (fFL) plotted as a function of L for constructs shown in panel a, with the SecM(Ms-Sup1) AP. The orange dot indicates the linker length chosen for mutagenesis experiments (L = 46 residues) Standard errors (s.e.m.) are shown. (c) Pulse labeling of the LepB[5D, L=46] construct (lane 1) and a corresponding control construct where the last proline residue in the AP has been mutated to alanine in order to render the AP non-functional (14) (lane 2). FL indicates the full-length and A the arrested form of the protein. An additional band representing a shorter form due to ribosomal stacking behind the stalled ribosome is indicated by *. B indicates a background band.

FIGURE 2. Mutagenesis scan of the SecM(Ms-Sup1) AP in the LepB[5D, L=46] construct. fFL values for all 228 single-residue mutations are shown. The sequence of the SecM(Ms-Sup1) AP is shown in larger font across the figure at its corresponding fFL = 0.56. Residue types are color coded: A, F, I, L, M, V, W, Y – orange; N, Q, S, T – blue; H, K, R – green; D, E – dark red; C, G, P – purple. Standard errors (s.e.m.) are not shown, but are similar in magnitude to the size of the individual letters (see Table S2).

FIGURE 3. Mutation of residues in AP positions -10 to -8 (red) in the LepB[10D, L=42], Ms-Sup1 construct to Trp. the 3W mutant has Trp in all three positions. Standard errors (s.e.m.) are shown.

FIGURE 4. fFL plotted as a function of L for the LepB[10D] construct with the SecM(Ms) AP (grey), SecM(Ms-Sup1) AP (blue), and the SecM(Ms-Sup1;S-9→W) AP (red). Standard errors (s.e.m.) are shown.

FIGURE 5. Analysis of the SecM(Ms-Sup1;S-2→P) AP. (a) SDS-PAGE analysis of the LepB[5D, L=42], SecM(Ms-Sup1; S-2→P) construct showing the presence of RNase-sensitive bands (bracketed). The original SecM(Ms-Sup1) AP is shown for comparison. Experiments are shown in triplicate. Full-length (FL) and arrested (A) forms of the protein are indicated. * indicates smaller forms caused by ribosome stacking behind the stalled ribosome. (b) Pulse-chase analysis of the LepB[10D, L=42], SecM(Ms-Sup1;S-2→P) construct. Non-radioactive methionine was added to the culture after a 2 min pulse with radioactive methionine. The RNase-sensitive bands are absent in this case. (c) Ala-scan of the SecM(Ms-Sup1; S-2→P) AP. Standard errors (s.e.m.) are shown.

FIGURE 6. fFL values for the LepB[5D, L=46], SecM(Ms-Sup1) construct where the codons for the indicated Ser residues (red) were changed to all six possible Leu codons. Standard errors (s.e.m.) are shown.
FIGURE 7. Oligo-proline stretches are strong APs in an $efp^-$ background. (a) Design of LepB[19A, $L=39$] and LepB[10D, $L=43$] constructs with oligo-proline APs, c.f. Fig. 1a. (b) Pulse labeling of LepB[19A, $L=39$] constructs with the SecM($Ms$) AP (lanes 1,2), with the SecM($Ms$) AP replaced by GSGSGGSS (lanes 3, 4), or with the last residue in the GSGSGGSS sequence replaced by P (lanes 5, 6), by PP (lanes 7, 8), and by PPP (lanes 9, 10). Proteins were expressed in the presence or absence of EF-P, as indicated. FL indicates the full-length and A the arrested form of the proteins. (c) Pulse labeling of the LepB[10D, $L=43$] construct with a PP stalling motif expressed in the presence or absence of EF-P as indicated. FL indicates the full-length and A the arrested form of the proteins. * indicates shorter protein fragments due to ribosome stacking behind the stalled ribosome. (d) $f_{FL}$ plotted as a function of $L$ for the LepB[10D] construct with the SecM($Ms$-Sup1) AP (blue), the SecM($Ms$-Sup1; S,9→W) AP (red), and an APPK stalling motif (green; construct expressed in an $efp^-$ strain). Standard errors (s.e.m.) are shown.

FIGURE 8. Analysis of the effects on $f_{FL}$ of residues immediately flanking a PP-stalling motif in the LepB[10D, $L=43$] construct expressed in $efp^-$ cells. (a, b) Results for positions $X$ and $Z$ in an $XPPZ$ motif. (c, d) The results for positions $X$ and $Z$ from Ref. 20 in the main text are plotted against the data from panels a and b, respectively. Standard errors (s.e.m.) are shown.

FIGURE 9. $f_{FL}$ values for the LepB[10D, $L=43$], SPPK construct expressed in $efp^-$ cells, with residues in positions -7 to -4 mutated to Trp, Arg, Pro, and Asp. Standard errors (s.e.m.) are shown.
Figure 1

(a) Diagram showing the membrane topology of a protein with transmembrane segments (TM1, TM2) and cytoplasmic (N) and periplasmic (C) domains.

(b) Graph showing the relationship between [kDa] and $f_{FL}$.

(c) Western blot analysis with bands at 35 kDa and FL, A, and B labels.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

a

b

Periplasm
Cytoplasm

SG-2P
SG-3P

MsAP
AP, PP, PPP

P

FL

A

EF-P

FL

A

EF-P

0.0
0.2
0.4
0.6
0.8
1.0

20
25
30
35
40
45
50
55

fL

SG-2P 10D
Figure 8
Figure 9
