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Effects on translation pausing of alterations in protein and RNA components
of the ribosome exit tunnel

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1 Abstract

2
3 Amino acids are polymerized into peptides in the peptidyl transferase center of the
4 ribosome. The nascent peptides then pass through the exit tunnel before they reach the
5 extra-ribosomal environment. A number of nascent peptides interact with the exit tunnel
6 and stall elongation at specific sites within their peptide chain. Several mutational
7 changes in RNA and protein components of the ribosome have previously been shown to
8 interfere with pausing. These changes are localized in the narrowest region of the tunnel,
9 near a constriction formed by ribosomal proteins L4 and L22. To expand our knowledge
10 about peptide-induced pausing, we performed a comparative study of pausing induced by
11 two peptides, SecM and a short peptide, Crb^{CmlA}, that requires chloramphenicol as a co-
12 inducer of pausing. We analyzed the effects of 15 mutational changes in L4 and L22, as
13 well as the effects of methylating nucleotide A2058 of 23S rRNA, a nucleotide
14 previously implicated in pausing and located close to the L4-L22 constriction. Our
15 results show that methylation of A2058 and most mutational changes in L4 and L22 have
16 differential effects on pausing in response to Crb^{CmlA} and SecM. Only one change, a six
17 amino acids insertion after amino acid 72 in L4, affects pausing in both peptides. We
18 conclude that the two peptides interact with different regions of the exit tunnel. Our
19 results suggest that either the two peptides use different mechanisms of pausing or they
20 interact differently but induce similar inhibitory conformational changes in functionally
21 important regions of the ribosome.

1 **Introduction**

2

3 Peptide bond formation during translation takes place within the large subunit of
4 the ribosome, at the peptidyl transferase center (PTC). In order to escape the ribosome,
5 newly formed peptides must traverse a 100Å long channel referred to as the peptide exit
6 tunnel. This tunnel is lined primarily by segments of 23S rRNA, although two ribosomal
7 proteins, L4 and L22, also contribute part of the tunnel lining (1, 26). These two r-
8 protein components form a constriction that results in the narrowest passage in the tunnel
9 (Fig. 1). Mutational changes in both rRNA and r-protein components located near the
10 constriction are important for bacterial resistance to macrolide-lincosamide-streptogamin
11 (MLS) antibiotics. These antibiotics bind to nucleotides A2058 and 2059 and other
12 close-by portions of 23S rRNA (reviewed in (11)).

13 The tunnel constriction has also been implicated in peptide-mediated pausing (7,
14 21). Peptide-mediated pausing occurs when sequence-specific interactions between the
15 nascent peptide and the translating ribosome cause a stall in translation. SecM is a well-
16 characterized example of a pause-inducing peptide. Mutations affecting the efficiency of
17 SecM-induced pausing are found in the codons for a 17 amino acids stretch of the
18 peptide, termed the pause motif (21). This region is sufficient for inducing pausing when
19 inserted into another protein sequence (10, 21). The SecM pausing response is also
20 decreased by mutations altering the ribosome. Two of these changes, an A to G change at
21 nucleotide 2058 in 23S rRNA and a three amino acid deletion in r-protein L22 (L22-
22 ΔMKR), are also known to confer resistance to erythromycin (4, 23, 32, 36). In addition,
23 several other L22 mutants shown to be deficient in SecM-mediated pausing do not confer

1 Ery resistance (21). These previous studies, implicating r-protein L22 and nucleotide
2 A2058 of 23S rRNA in SecM-mediated pausing, suggest that the region around the
3 tunnel constriction is important for this pausing event. However, the well-studied K63E
4 change in ribosomal protein L4, also located at the constriction, does not affect SecM-
5 mediated pausing, although it does bestow resistance to erythromycin [(21) and this
6 report].

7 Having recently isolated new erythromycin resistant mutations in the genes for
8 ribosomal proteins L4 and L22 of *E. coli* (39), we wanted to take a more in-depth look at
9 the relationship between erythromycin resistance and the efficiency of SecM-mediated
10 pausing in these mutants. In addition, we wanted to expand this analysis to another class
11 of pause-inducible peptides, those requiring an antibiotic for induction of genes
12 bestowing resistance to the inducing antibiotic. CmlA is one such system. Pausing in this
13 system is mediated by a leader peptide (Crb^{CmlA}) encoded by a nine codon open reading
14 frame upstream of the *cmlA* gene (8, 28). Unlike the SecM system, pausing mediated by
15 the Crb^{CmlA} peptide requires, as a co-factor, sublethal levels of chloramphenicol, the drug
16 to which the *cmlA* protein product confers resistance (2, 3). Pausing positions the
17 ribosome on the mRNA to prevent formation of a hairpin that, in the absence of a paused
18 ribosome, occludes the *cmlA* translation initiation site. The result is chloramphenicol
19 induced synthesis of CmlA.

20 Our results indicate that all of the new EryR mutational changes in L22 are
21 deficient in SecM-mediated pausing, but none has a significant effect on
22 chloramphenicol-induced Crb^{CmlA}-mediated pausing. On the other hand, the mutational
23 changes in L4 reduced Crb^{CmlA}-mediated pausing, but only one mutant demonstrated a

1 significant deficiency in SecM pausing. We also found that methylation of A2058 in 23S
2 rRNA significantly reduces Crb^{CmlA}-mediated pausing, but has no effect on recognition
3 of SecM. Our results suggest the SecM and Crb^{CmlA} peptides induce pausing by
4 interacting with different features of the exit tunnel.

5

6

7 **Materials and Methods**

8

9 *Primers Used*

10 See Table 1.

11

12 *Strains and Plasmids*

13 Strains used in this study were derived from DY380, a DH10B derivative
14 containing a λ prophage encoding phage proteins for homologous recombination (16).
15 Strains were constructed as follows: DNA sequences encoding pausing peptides were
16 amplified from the chromosome of *E. coli* K12 for the *secM/lacZ* construct (primers
17 O1293 and O1294, Table 1) and from plasmid pDU1294 [described in Dorman and
18 Foster (8)] for the *crb^{cmlA}/lacZ* construct (primers O1893 and O1894, Table 1). The PCR
19 fragments containing the pause sequences were cloned into pGEM5 to create in-frame
20 fusions with *lacZ* α . SacI and MluI sites were used for SecM, while SpeI and NotI were
21 used for Crb^{CmlA}. These pause sequence/*lacZ* α fusions were then PCR amplified (O1940
22 and O1941, Table 1) and inserted into the chromosomal *lacZ* gene by homologous
23 recombination. For the *secM/lacZ* fusion, we first constructed a Lac⁺ derivative of

1 DY380 by converting its chromosomal *lacZ* Δ M15 gene to wildtype *lacZ* using a PCR
2 product derived from the *lacZ* α gene of pGEM5. The latter was synthesized using
3 primers O1940 and O1941. We then replaced by recombineering the N-terminal end of
4 *lacZ* with the *secM/lacZ* fusion on pGEM5, also synthesized using primers O1940 and
5 O1941, and screened for white colonies. In the resulting strain, called DY380(SecM), the
6 *lacZ* gene on the chromosome is replaced by an open reading frame with the following
7 sequence: N-terminal 5 codons of *lacZ*, 18 codons from pGEM5, 46 codons from *secM*
8 (Thr121 through Pro166), 39 codons from pGEM5, and the rest of *lacZ* (beginning with
9 codon 6) (Fig. 2A). We inserted the *crb^{cmlA}/lacZ* fusion into DY380 (containing
10 *lacZ* Δ M15) and screened for blue colonies. The resulting strain, called DY380(CmlA),
11 has on the chromosome an open reading frame containing the N-terminal 5 codons of
12 *lacZ*, 30 codons from pGEM5, and the entire *crb* sequence (10 codons including the
13 termination codon), followed by the natural intergenic spacer between *crb* and *cmlA*, a
14 second open reading frame containing the N-terminal 15 codons of *cmlA*, 27 codons from
15 pGEM5, and *lacZ* starting with codon 6 (Fig. 3A).

16 L4 and L22 mutations were introduced into the chromosomes of DY380(SecM)
17 and DY380(CmlA) by homologous recombination using PCR products generated with
18 primers O1460 and O1461 for L22 and O1462 and O1455 for L4 (Table 1). For
19 erythromycin resistant mutations (39), we selected recombinants on LB plates containing
20 400 μ g/ml of erythromycin. Non-resistant mutants L22-G91A and L22-G91D were
21 identified on the basis of their Lac⁺ phenotype. The L22- Δ loop2 mutant was also
22 identified as a Lac⁺ recombinant; in this mutant, amino acids 82-98 of the L22 tentacle

1 have been replaced with two glycines (also, an unintentional base change in codon 101
2 changed Ser to Gly) (40).

3 The sequences of the *lacZ* fusions and mutant L4 and L22 sequences were
4 confirmed by sequencing using ABI Big Dye v3.

5 Plasmid pErmC was constructed by amplifying the *ermC* gene from pE194 (15)
6 (obtained from the Bacillus Genetics Stock Center, BGSC No. 1E7), using primers
7 O1862 and O1871 (Table 1) and cloning into pBAD18 using *EcoRI* and *KpnI*. In the
8 resulting construct, ErmC expression is induced in the presence of arabinose.

9 Plasmids used to express L22 under arabinose control were described previously
10 (40).

11

12 *β -galactosidase assays*

13 β -galactosidase assays were performed on cultures grown at 30°C in LB media
14 containing 1mM IPTG. Cultures were inoculated at OD600 between 0.04 and 0.05, and
15 grown at 30° to an OD600 of 0.4 to 0.6 (approximately 8×10^7 cells/ml). Where
16 indicated, arabinose (0.2% final concentration) was used to induce ErmC synthesis.

17 Crb^{CmlA}-mediated translation pausing was analyzed by growing cells in the presence of
18 chloramphenicol (0.8 μ g/ml). Enzyme assays were performed basically as described
19 (19), and read in a 96-well microtiter dish using a plate reader (12).

20

21 *Plate screen for SecM deficient mutants*

22 Wildtype DY380 or DY380(SecM) containing plasmids with wildtype or mutant
23 L22 downstream of the arabinose-inducible pBAD promoter were grown on LB solid

1 media containing 1 mM IPTG, 0.2% arabinose and ampicillin at 200 μ g/ml. Plates were
2 incubated at 30°C and visually analyzed for colony color. Wildtype DY380 formed
3 white colonies under these conditions, while colonies deficient for SecM pausing were
4 varying degrees of blue.

6 *Ribosome Preparations*

7 Cells were grown to an $OD_{450} = 1.5-2.0$ (about 3×10^8 cells per ml), harvested on
8 ice, and centrifuged at 8K for 10 min in a Beckman JLA 10.5 rotor. Cells were then
9 resuspended in Buffer A (20 mM HEPES-KOH pH 7.5, 6 mM $MgCl_2$, 30 mM NH_4Cl , 6
10 mM β -mercaptoethanol) and lysed using a French press at 16,000 psi. Lysates were
11 clarified by spinning at 22000 rpm for 30 min in a Beckman MLA-80 rotor, and
12 ribosomes were pelleted from the supernatant by spinning at 50000 rpm for 4 hours. The
13 ribosome pellet was washed with buffer A and resuspended overnight in 400 μ l buffer A.
14 Ribosome were salt-washed by mixing 1 part crude ribosomes and 9 parts salt-wash
15 buffer (20 mM HEPES-KOH pH 7.5, 6 mM $MgCl_2$, 1M NH_4Cl , 6 mM β -
16 mercaptoethanol), incubating on ice for 1 hour, and centrifugation at 50000 rpm for 4
17 hours. Ribosomes were resuspended as described above.

19 *Erythromycin Binding*

20 Binding of erythromycin was determined as described in Zaman et al. (39). This
21 method is modified from (30).

23 *ErmC induction and Analysis of A2058 Methylation*

1 Cells containing pErmC were grown to mid-log phase in the presence or absence
2 of 0.2% arabinose. Cultures were then harvested and ribosomes prepared as described
3 above. rRNA was phenol-chloroform-extracted from purified ribosomes followed by two
4 ethanol precipitations. Methylation of A2058 was quantified using previously published
5 methods (27, 33). Briefly, primer O2040 (Table 1) was 5'-³²P-end labeled and
6 hybridized to 5 µg purified rRNA. The primer was extended in the presence of dTTP and
7 ddCTP using reverse transcriptase. Reverse transcriptase reactions were then phenol-
8 chloroform-isoamylalcohol extracted, chloroform extracted, ethanol precipitated, and
9 resuspended in 15 µl of H₂O. Four µl were then run on a 20% denaturing polyacrylamide
10 gel, and the gel was dried and then visualized on a phosphorimager (Molecular
11 Dynamics). Band intensities were quantified using Image Quant software.

14 Results

16 *Effect of L22 mutants on SecM-mediated pausing*

17 For many years, only one mutation in the L4 gene and one mutation in the L22
18 gene had been shown to confer resistance to erythromycin in *E. coli*. We recently
19 reported the isolation of new erythromycin-resistant ribosomal protein mutants (39).
20 Eight mutations mapped in the gene for r-protein L4 and two mapped in L22. We were
21 interested in determining if these new EryR ribosomal protein mutational changes affect
22 SecM pausing.

1 We constructed a derivative of strain DY380 (16) in which the SecM pause
2 sequence is fused to the N-terminus of the gene encoding β -galactosidase, resulting in the
3 strain referred to as DY380(SecM) (Fig. 2A). Mutations in the ribosomal protein L22
4 gene conferring erythromycin resistance, originally isolated in *E. coli* strain AB301, were
5 introduced into the chromosome of DY380(SecM) by homologous recombination and
6 selected on erythromycin solid media. β -galactosidase assays were performed on both
7 wildtype and mutant strains to measure the effect of L22 mutations on *secM/lacZ*
8 expression. In this system, pausing directly affects the expression of the *lacZ* fusion
9 protein (Fig. 2A). Thus, increased β -galactosidase activity indicates that ribosomes
10 carrying a mutant protein are deficient in SecM pausing.

11 The original EryR L22 mutant (which we call L22- Δ MKR) has a deletion that
12 removes amino acids 82 to 84 from the so-called “tentacle”, the region of the protein that
13 lines the peptide exit tunnel (31). One of the new mutants, L22-99/+15, has a 15-amino
14 acid insertion after amino acid 99, also in the tentacle. The other new L22 mutant, L22-
15 105/+2, has a two amino acid insertion after amino acid 105; this insertion is just
16 downstream of the tentacle. Like L22- Δ MKR, both of our new L22 EryR mutants
17 accumulated significantly more β -galactosidase than wildtype DY380(SecM) (Fig. 2B),
18 indicating that all three EryR strains are defective in responding to the SecM pause
19 signal.

20 As a comparison, we also introduced two other mutations into the L22 gene that
21 had previously been shown to result in defective SecM pausing but not resistance to
22 erythromycin (21). These mutants, L22-G91A and L22-G91D, also accumulated more β -
23 galactosidase than the parent strain (Fig. 2B), although much less than the EryR mutants.

1 We also isolated two other mutants from DY380(SecM) that appeared spontaneously as
2 bluer colonies on X-gal plates and, upon sequencing, were found to have changes in L22:
3 L22-R84L and a mutant containing two alterations, L22-A89V and R94H. As predicted
4 from their plate phenotype, these two mutants also accumulated more enzyme than the
5 parent (Fig. 2B and data not shown). These mutants were not resistant to Ery, and, like
6 the original EryS G91A and G91D mutants, were not as defective in SecM pausing as the
7 EryR mutants.

8 We have previously reported that L22 proteins containing deletions removing
9 most or all of the tentacle are nevertheless still able to be incorporated into ribosomes that
10 can be assembled into polysomes (40). As an initial screen of the effect these deletions
11 have on the SecM response, we introduced into strain DY380(SecM) plasmids carrying
12 two different tentacle-deletion L22 genes. L22- Δ loop1 lacks amino acids 85 – 95, and
13 L22- Δ loop2 lacks amino acids 82 – 98; both genes are under control of the arabinose-
14 inducible PBAD promoter (40). Cells expressing these mutant proteins from plasmids
15 were also defective in SecM pausing, as judged by bluer colonies on X-gal plates
16 compared to cells expressing wildtype L22 (data not shown). To confirm these results,
17 we substituted the wildtype chromosomal copy of L22 with the L22- Δ loop2 tentacle
18 deletion derivative. The resulting mutant had a significant defect in SecM-mediated
19 pausing (Fig. 2B). It was also resistant to erythromycin, although maximal erythromycin
20 concentration tolerated by the mutant was only 100 μ g/ml, compared to 200 μ g/ml or
21 more for our other L22 EryR mutants. The isolation of this mutant strain, now harboring
22 a single L22 gene encoding a protein with no tentacle, confirms our previous report that
23 the L22 tentacle is not necessary for ribosome function (40).

1 It should be noted that, although the L22 mutants just described had significantly
2 higher levels of β -galactosidase than the wildtype parent, the enzyme levels were still
3 much lower than the level we observed with a strain containing a defective SecM signal
4 fused to *lacZ*. In this mutated pause sequence, a proline to alanine change at position 166
5 of SecM abolishes the pausing activity of SecM (21). This construct (SecM-P166A)
6 accumulated more than 10 times as much β -galactosidase activity as the most defective
7 L22 strain (Fig. 2B). We conclude that all of the L22 mutants tested here pause less
8 efficiently in response to SecM than wildtype cells, although they still show significant
9 pausing.

11 *Effect of L4 mutants on SecM-mediated pausing*

12 We also introduced the canonical L4 erythromycin resistant mutation (L4-K63E)
13 and seven new L4 mutations into strain DY380(SecM). All of these mutations map in the
14 region of the gene encoding the tentacle of L4, the tip of which forms part of the lining of
15 the peptide exit tunnel (34). Unlike the L22 EryR mutations, mutations in the L4 gene
16 had little or no effect on the cell's response to the SecM pause signal (Fig. 2B). The
17 amino acid substitution mutants were essentially like the wildtype parent. The three
18 insertion mutants showed slightly higher β -galactosidase activity, but only mutant L4-
19 72/+6, which has a six amino acid insertion after amino acid 72 of L4, showed increased
20 β -galactosidase activity, comparable to the EryS L22 mutants. This is the first mutation
21 in the L4 gene reported to affect the ribosome's response to SecM.

23 *Effect of L4 and L22 EryR mutants on pausing induced by the leader peptide of CmlA*

1 Translation of the leader upstream of CmlA causes ribosomal pausing in the
2 presence of sub-lethal levels of chloramphenicol (2, 8, 17). We wanted to compare the
3 effects of EryR mutants on SecM-mediated pausing with their effects on
4 chloramphenicol-induced pausing mediated by the CmlA leader peptide. Therefore, we
5 constructed a strain in which the leader peptide (Crb^{CmlA}), the intercistronic region
6 between the Crb leader and *cmlA*, and first 15 codons of *cmlA* were inserted into the *lacZ*
7 gene present on the chromosome, creating an in-frame fusion between *cmlA* and *lacZ*
8 (Fig. 3A). Since pausing of ribosomes at a specific position in the Crb leader prevents
9 the formation of the hairpin in the *crb-cmlA* intercistronic region, pausing exposes the
10 *cmlA* translation initiation site and stimulates CmlA translation (28). Thus, the CmlA
11 reporter differs from the SecM reporter in that decreased pausing results in decreased β -
12 galactosidase activity. In the DY380(CmlA) construct, the Crb pausing sequence is
13 moved from its natural state as a short 9 amino acid open-reading frame upstream of
14 *cmlA* to a position 36 codons from the translation initiation site, yet the sequence still
15 displayed efficient pausing (Fig. 3A).

16 Contrary to the results with SecM, we found that, in the presence of
17 chloramphenicol, the mutational changes in L22 had little or no effect on *cmlA-lacZ*
18 expression. On the other hand, most of the L4 mutants exhibited reduced levels of β -
19 galactosidase activity and, hence, reduced responses to Crb^{CmlA}-mediated pausing (Fig.
20 3B). The three amino acid substitutions at Gly-66 (G66D, G66R, and G66S) resulted in
21 the weakest effects. More significant reductions were seen with L4 mutants Q62K,
22 K63E, 56/+6 (6 amino acid insertion after amino acid 56), and 63/+4 (4 amino acid
23 insertion after amino acid 63), which yielded β -galactosidase levels that were between 60

1 and 70% of the wildtype level. The greatest defect in Crb^{CmlA}-mediated pausing was
2 observed with the mutant L4-72/+6, which was induced by chloramphenicol to only half
3 the wildtype level. Interestingly, this strain was the only one that exhibited defects with
4 both pausing systems.

5 In the absence of chloramphenicol, all of the L4 mutants, except L4-72/+6,
6 produced about twice as much β -galactosidase as the wildtype parent and the L22
7 mutants. As a result, the induction ratios for the L4 mutants ranged from 15- to 24-fold,
8 while the wildtype parent and L22 mutants had induction ratios ranging from 43- to 85-
9 fold. The relatively higher levels of β -galactosidase synthesis in the absence of
10 chloramphenicol (compared to the wildtype parent) suggest that the mutational changes
11 do not have a general inhibitory effect on β -galactosidase synthesis that could account for
12 the reduced β -galactosidase levels after addition of the antibiotic. Consistent with this,
13 we observed that mutational changes in L4 that resulted in reduced chloramphenicol
14 induction levels had no effect on expression of a wildtype *lacZ* gene on the chromosome
15 after induction with isopropyl β -D-1-thiogalactopyranoside (IPTG) (data not shown).
16 We also considered the possibility that chloramphenicol itself may have an inhibitory
17 effect on synthesis of β -galactosidase in strains with mutated L4 genes, independent of its
18 effect on stalling. However, addition of chloramphenicol at the sublethal concentrations
19 used to induce pausing showed that the level of enzyme encoded by the IPTG-induced
20 chromosomal *lacZ* gene was not affected by mutational alterations in L4 (data not
21 shown). Finally, the deficiencies in Crb pausing are not due to increased resistance to
22 chloramphenicol, since only the L4 G66D mutation resulted in increased (but slight)
23 resistance to chloramphenicol (results not shown). We conclude that the decrease in β -

1 galactosidase expression seen in the L4 strains compared to the wildtype and L22 strains
2 reflects a decreased ability to respond to the Crb^{CmlA} stalling peptide.

3

4 *Methylation of A2058 and Pausing*

5 Previous studies have shown that an A to G mutation at nucleotide 2058 in 23S
6 ribosomal RNA reduces the response to SecM-mediated pausing (21). The same
7 mutation also eliminates the inhibitory effects of MLS drugs (9, 32). Methylation of
8 A2058 by the ErmC methylase also confers resistance to erythromycin (35). To test if
9 ErmC methylation affects SecM-mediated pausing, we constructed a plasmid in which
10 the gene encoding *ermC* is placed under control of the inducible araBAD promoter; in
11 this way the methylase is expressed only in the presence of arabinose. We confirmed
12 methylation of A2058 in the presence of arabinose by reverse transcription of rRNA
13 extracted from purified ribosomes (Fig. 4A); these experiments indicate that
14 approximately 80% of the 23S rRNA molecules were methylated when *ermC* was
15 induced (Fig. 4B). As expected, methylation strongly reduced binding of erythromycin
16 to ribosomes (Fig. 4C). Strain DY380(SecM) containing this plasmid was then assayed
17 for β -galactosidase activity in the presence and absence of arabinose. We found that,
18 unlike the A2058G rRNA mutant, which exhibits decreased pausing (21), methylation of
19 A2058 had no significant affect on SecM-mediated pausing (Fig. 4D). In contrast,
20 arabinose induction of pErmC in the strain carrying the Crb^{CmlA} pause system
21 DY380(CmlA) reduced β -galactosidase activity by 60%, indicating that methylation of
22 A2058 in 23S rRNA significantly inhibits chloramphenicol-induced Crb^{CmlA} activity

1 (Fig. 4E). Strains carrying the empty vector or expressing a control protein did not show
2 this defect in the presence or absence of arabinose (data not shown).

4 **Discussion**

5 Regulation of translation by cis-acting pause-inducing peptides involves
6 interactions between the nascent peptide and ribosomal components lining the peptide
7 exit tunnel (6, 7, 14, 17, 21). The precise mechanisms by which these interactions inhibit
8 subsequent peptidyl transferase activity are still unclear. Previous studies of the SecM
9 and TnaC systems implicated the region of the tunnel where a constriction is formed by r-
10 proteins L4 and L22 (7, 21), but differential effects of ribosomal mutations suggested that
11 the two systems may not work by exactly the same mechanism (18). In this work, we
12 have shown that a large collection of L4 and L22 mutants as well as the methylation of
13 the 23S rRNA nucleotide A2058 affect pausing in response to SecM and another pausing
14 peptide, Crb^{CmlA}. However, with the exception of one L4 mutant, we observe complete
15 non-overlap in the effects of the mutations on one system or the other. Our results not
16 only add to our growing knowledge of the role played by ribosomal components near the
17 constriction in the function of regulatory peptides, but also highlight apparent differences
18 in the mechanism by which pause-inducing peptides act.

20 *Limited effects of L22 mutations*

21 It is important to note that the effects on SecM-mediated pausing attributed to
22 mutations in the L22 gene are relatively small compared to the effect observed in cells
23 carrying the P166A substitution in the SecM pausing peptide (numbering relative to the

1 intact SecM protein), which eliminates pausing altogether (21). In the SecM system, the
2 greatest effects of changes in the L22 protein resulted in accumulation of less than 10%
3 the β -galactosidase activity observed with the P166A mutation in the pausing peptide.
4 The ribosomal mutants are thus not completely devoid of pausing function. If the ability
5 to recognize specific signals in nascent peptides is a critical and integral part of ribosome
6 function, perhaps a ribosome completely oblivious to a pause signal would be too
7 dysfunctional to support growth. This idea is consistent with our observation that the
8 L22 mutants we described here are able to support growth at doubling times no more than
9 four-fold greater than the wildtype growth rate ((39) and data not shown)).

11 *Changes in L22 and L4 affect SecM and Crb^{CmlA} differently*

12 Previous studies have identified ribosomal protein L22 as particularly important
13 to the activity of both SecM and TnaC pausing peptides (7, 21). The experiments
14 reported here show that mutations in the L22 gene that bestow erythromycin resistance
15 (Δ MKR, 99/+15, 105/+2, and Δ loop2) have significantly stronger effects on pausing than
16 do mutations with no detectable change in the level of erythromycin sensitivity (G91A,
17 G91D, and R84L). Presumably, mutations that confer resistance to erythromycin have a
18 more severe conformational effect on the region(s) of the tunnel involved in SecM
19 recognition and Ery inhibition. However, our experiments cannot determine whether the
20 same tunnel determinants are involved in both.

21 Contrary to the SecM peptide (21, 38), pausing mediated by the Crb^{CmlA} peptide is
22 affected by alterations in the L4 protein, but not by mutations altering L22. Another
23 difference between the two pausing peptides is that only Crb^{CmlA}-mediated pausing

1 requires a co-inducer, chloramphenicol. It would seem that chloramphenicol-induced
2 alterations in ribosome structure must be combined with peptide-induced changes in
3 order to establish the pausing condition.

4

5 *Mechanism of pausing*

6 All but one (L4-72/+6) of the L4 mutants showed increased basal level expression
7 of the CmlA/ β -galactosidase fusion protein when compared to wildtype or L22 mutants,
8 indicating an increase in the amount of time ribosomes occupied the Crb^{CmlA} pause site in
9 these mutants. Possibly, these elevated basal levels result from an increased sensitivity of
10 the mutant ribosomes to the Crb^{CmlA} pause sequence in the absence of the inducer
11 chloramphenicol. This would agree with previous reports that a synthetic peptide of the
12 Crb^{CmlA} 8-mer is able to induce changes in ribosome structure *in vitro* in the absence of
13 chloramphenicol (14). If certain L4 mutants are indeed more sensitive to the Crb^{CmlA}
14 peptide alone, it is not clear why these same mutants show reduced levels of induction in
15 the presence of chloramphenicol. It is not likely that these mutations inhibit binding of
16 the drug at its normal binding site, since only one of the L4 mutants exhibiting increased
17 basal levels of CmlA/ β -gal (G66D) also showed an increased resistance to
18 chloramphenicol. It was previously shown that chloramphenicol-resistant mutations do
19 not impair Crb^{CmlA}-mediated pausing and, conversely, mutations that impair pausing do
20 not affect chloramphenicol's inhibitory effects (24). Our results in conjunction with
21 these previously published observations suggest that chloramphenicol may bind
22 differently to the ribosome during general inhibition of protein synthesis and during
23 Crb^{CmlA}-mediated pausing.

1 Our genetic experiments agree with previous reports indicating that the
2 constriction in the vicinity of A2058 and the tips of L4 and L22 (Fig. 5) is important for
3 translation pausing induced by the nascent peptide. However, if the constriction indeed
4 does gate the tunnel as previously suggested (21), the L22 tentacle is not likely an
5 essential part of this gate. This conclusion is based on the observation that deletion of the
6 entire L22 tentacle only mildly affects pausing. It is important to note that the L22-
7 Δ loop2 gene in the strain used in the Figs 2 and 3 is in the chromosomal S10 operon and
8 hence is the only source of L22. This is different from our previous construct in which
9 the strain contained a wildtype L22 gene in addition to the plasmid-based L22- Δ loop
10 mutant gene (40). Based on the experiments reported here, we can therefore
11 unambiguously conclude that the L22 tentacle is not essential for protein synthesis or
12 pausing. Moreover, the haploid L22- Δ loop2 mutant grows only a little slower than the
13 wildtype parent (data not shown), indicating that ribosomes carrying the tentacle-less L22
14 function well in protein synthesis.

15 The positions of the various L22 mutations indicate that for at least some of the
16 mutations, the effects are indirect. Perhaps the most potent argument for this idea is that
17 the complete deletion of the L22 tentacle, which contributes to the tunnel lining over
18 almost 2/3 of the tunnel length (Fig. 5), has no stronger an effect on SecM pausing than
19 do the other L22 mutations. If direct interaction(s) between the pausing peptide and L22
20 were essential, we would have expected that the deletion of the L22 tentacle should have
21 eliminated pausing entirely. Furthermore, a mutation in the globular portion of L22
22 outside the tunnel (105/+2) affects pausing more severely than point mutations within the
23 tentacle located in the tunnel.

1 The idea that ribosomal protein mutations can have indirect effects on tunnel
2 function also arises from the short length of the Crb^{CmlA} pause peptide, which is only
3 eight amino acids long. If this peptide were fully extended, it could reach as far as about
4 25Å from the PTC (assuming 3-3.4Å per residue). If the nascent Crb^{CmlA} peptide forms a
5 more helical structure, it might reach only as far as 12Å (assuming 1.5Å per residue).
6 That range of reach suggests that the nascent peptide could interact with nucleotide
7 A2058, but it is not likely to form an extensive interaction with the L4/L22 constriction,
8 which is 20-35Å from the PTC (18) (Fig. 5). Thus, it seems inescapable that the effects
9 of the L4 mutants on Crb^{CmlA}-mediated pausing are indirect. Indirect effects of mutations
10 on ribosome function is also compatible with effects of the L4-K63E mutation on 50S-
11 mediated peptidyl transferase activity and 30S-mediated decoding (22, 36); both of these
12 activities involve regions of the ribosome that are distant from amino acid 63 of L4.
13 Several other L4 and L22 mutations, located across extensive portions of the ribosome
14 and none of which are located close to the PTC or other ribosome functional centers, also
15 affect the peptide growth rate of peptide chain elongation (39). Furthermore, a two amino
16 acid deletion in *Streptococcus pneumoniae* L4 results in resistance to not only
17 erythromycin, but also chloramphenicol (37), which binds closer to the PTC than
18 erythromycin (25) (Fig. 1).

19 The SecM peptide, which is 17 amino acids long, has the potential to form
20 interactions in a much more extended ribosome landscape than does the 8 amino acid
21 Crb^{CmlA} peptide, even though the SecM peptide apparently condenses during pausing
22 (38). Interestingly, the six C-terminal amino acids of the SecM peptide are sufficient to
23 induce a pause (5, 29), albeit not as efficiently as that caused by full length SecM. Thus,

1 interactions in the tunnel close to the PTC may establish a minimal pause. It has been
2 hypothesized that essential interactions between the C-terminal portion of SecM and the
3 ribosome tunnel between the PTC and A2058 are enhanced by interactions between the
4 N-terminal part of the SecM pausing peptide and L22 in the more distant portions of the
5 tunnel (21). Perhaps chloramphenicol in some way substitutes for the effect of the N-
6 terminal portion of the SecM pausing peptide in facilitating Crb^{CmlA}-mediated pausing.

7 Previous reports have proposed that interaction between SecM and nucleotide
8 A2058 of 23S rRNA is important for SecM-mediated pausing (20, 21). While the SecM
9 peptide may interact with A2058, as suggested by the effect of the A2058G mutation, any
10 putative interaction does not mimic the interaction of erythromycin with A2058, since we
11 showed that methylation of A2058 by the ErmC methylase inhibits the binding of
12 erythromycin, but has no effect on SecM activity. The difference between the effects of
13 A2058G mutation and methylation of the same nucleotide on SecM-mediated pausing
14 suggests that either SecM and erythromycin interact differently with this nucleotide, or
15 that the effects of the A2058G mutation on SecM pausing are indirect.

16 It has been reported that methylation of A2058 has no effect on the activity of
17 another chloramphenicol-induced regulatory peptide, Cat-86 (13), a pausing peptide
18 related to Crb^{CmlA}. However, our results show that methylation of A2058 dramatically
19 reduces Crb^{CmlA}-mediated pausing. While Cat-86 only has a length of five amino acids as
20 opposed to eight for Crb^{CmlA}, it is quite intriguing that two peptides which are regulated
21 by the same drug would have different responses to the same rRNA modification. This
22 could indicate that Crb^{CmlA} and Cat-86 interact differently with the ribosome and
23 therefore possibly have different mechanisms of inhibition.

1 In summary, the comparison of SecM and Crb pausing reveals new diversity in
2 the mechanisms of nascent-peptide-induced inhibition of PTC function. Further studies
3 should contribute not only to our understanding of pausing, but of ribosome assembly and
4 function in general.

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1 **Legends to Figures**

2

3 Fig. 1. Cartoon model of 50S subunit of the ribosome of *E. coli*, showing the peptidyl
4 transferase center (PTC), the peptide exit tunnel, the tips of r-proteins L4 and L22, and
5 the approximate binding sites of antibiotics chloramphenicol (CM) and erythromycin
6 (Ery).

7

8 Fig. 2. SecM-mediated pausing in L4 and L22 mutants. A. Map of construct for
9 quantitating efficiency of pausing. White boxed areas indicate sequences derived from
10 the multi-cloning site of pGEM5. The SecM sequence is shown in black. Light gray
11 boxes indicate sequences from the *lacZ* gene. For more details, see Materials and
12 Methods. B. Quantitation of SecM/ β -galactosidase fusion protein synthesis. The β -
13 galactosidase activity was measured in strains containing the indicated L22 or L4 genes,
14 or using a fusion protein construct containing a mutation in the SecM sequence (P166A)
15 that inactivates the pausing peptide (21), as described in Materials and Methods. At least
16 three independent cultures were analyzed for each mutant, and β -galactosidase assays
17 were performed in duplicate for each culture. The standard error of the mean is
18 indicated.

19

20 Fig. 3. Crb^{CmlA}-mediated pausing in L4 and L22 mutants. A. Map of construct for
21 quantitating efficiency of pausing. White boxed areas indicate sequences derived from
22 the multi-cloning site of pGEM5. This region is interrupted by the segment of DNA
23 containing the crb pausing peptide (black box), intergenic region, and the N-terminus of

1 *cmlA* (black box). Light gray boxes indicate sequences from the *lacZ* gene. For more
2 details, see Materials and Methods. B. Quantitation of CmlA/ β -galactosidase fusion
3 protein synthesis. The β -galactosidase activity was measured in strains containing the
4 indicated L22 or L4 genes, as described in Materials and Methods. The light gray bars
5 indicated measurements from cells grown in the absence of antibiotic. The black bars
6 indicate measurements from cells grown in the presence of chloramphenicol (0.8 μ g/ml).
7 At least three independent cultures were analyzed for each mutant, and β -galactosidase
8 assays were performed in duplicate for each culture. The standard error of the mean is
9 indicated for the induced values.

10
11 Fig. 4. Effect of methylation of A2058 on SecM- and Crib^{CmlA}-mediated pausing and
12 erythromycin binding. Wildtype cells containing a plasmid with an arabinose-inducible
13 ErmC gene were grown in the absence or presence of arabinose. Aliquots were removed
14 for quantitation of β -galactosidase activity, and the remainder of the cultures were used
15 for ribosome preparation and primer extension and erythromycin binding analysis (see
16 Materials and Methods for details). A. Secondary structure of 23S rRNA in the region of
17 A2058. The oligo used for primer extension was complementary to the highlighted
18 bases. B. Primer extension analysis of the methylation of A2058. P: primer; 2059:
19 extension products terminated at nucleotide 2059 (indicating methylation blockage at
20 A2058) and 2057: extension products terminated at G2057 (no methylation). C.
21 Binding of ¹⁴C-erythromycin to ribosomes from cells grown with and without arabinose.
22 D. Quantitation of SecM/ β -galactosidase fusion protein synthesis in cells grown with and
23 without arabiinose. Standard errors of the mean are indicated. E. Quantitation of

1 CmlA/ β -galactosidase fusion protein synthesis in cells grown in the presence of
2 arabinose. Standard errors of the mean are indicated.

3

4 Fig. 5. Structure of the *E. coli* ribosome exit tunnel. The figure shows a slab view
5 through the ribosome tunnel from the peptidyl transferase center (PTC) past the region of
6 the tunnel lined on one side by the L22 tentacle. Nucleotides A2450-A2451 (magenta)
7 are used as markers for the PTC. Also shown are A2058-A2059 (red), marking the
8 erythromycin binding site and the entry to the beginning of the narrow part of the tunnel.
9 Nascent peptides are synthesized at the PTC, then migrate past nucleotides A2058 and
10 A2059 and through the constriction formed by the tips of r-proteins L4 and L22 before
11 moving further into the tunnel lined on one side by the L22 tentacle. The tentacles of L4
12 (blue) and L22 (yellow) are shown with selected side chains marking positions of amino
13 acid substitutions, insertions and deletions analyzed in this study. Of particular interest is
14 the Δ loop2 deletion in L22, in which all residues from M82 through K98 were replaced
15 with two glycine residues. In this mutant, much of the L22 contribution to the tunnel
16 lining is presumably eliminated. For more details about mutants, see text and (39, 40).

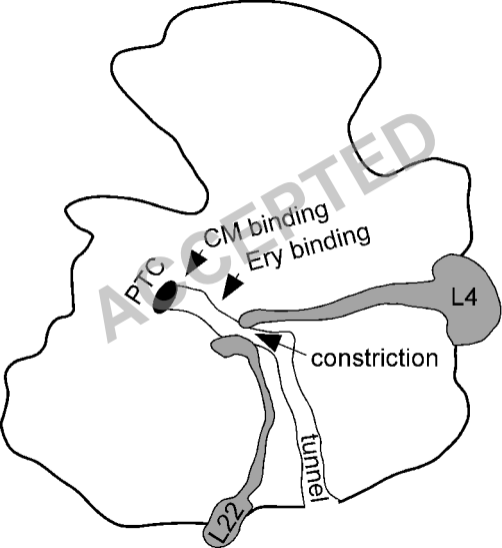
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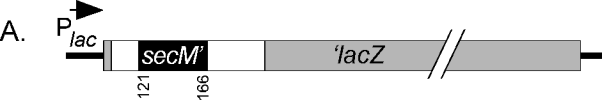
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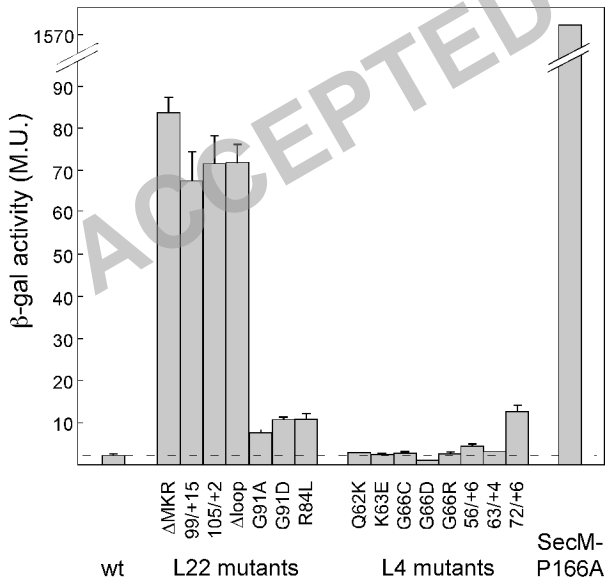
Table 1. Oligonucleotides used

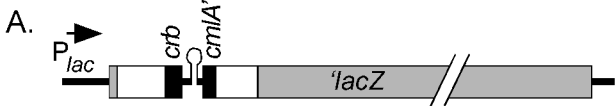
Oligo #	Name	Used to:	Sequence
O1460	L22 forward	Amplify, sequence or recombineer L22 gene	GAATTCGCACCGACTCGTAC
O1461	L22 backward		GGTGTTTCGCAAACCAGGTAGAG
O1462	L4 forward	Amplify, sequence or recombineer L4 gene	CTGGTTAAAGGTGCTGTCCC
O1455	L4 backward		CCATCGCAGTAGACGCTTTTTTC
O1893	Crb ^{CmlA} forward	Amplify, clone <i>crb^{CmlA}-cmlA</i> '	CCCCGCGGCCGCGTTACGATTCAAATTCAATCATGAGAT
O1894	Crb ^{CmlA} backward		CCCCACTAGTCGTGGCGGCAAGGGAGTACCGCCAACTAAA
O1293	<i>secM</i> forward	Amplify, clone <i>secM</i> pausing motif	ATTTCCAACGCGTTGACGCTCAGCGCGCTGCTGAC
O1294	<i>secM</i> backward		CGATGAGAGCTCCAGGGCCAGCACGGATGCCTTGC
O2040	Complement 23S 2061-2078	Measure methylation of 2058	GTAAAGGTTACGGGGTC
O1862	<i>ermC</i> forward EcoRI	Amplify, clone <i>ermC</i>	TCTTCTGAATTCAGGAGGTCTTCTTCTATGAACGAGAAAAA
O1871	<i>ermC</i> backward XbaI		TATAAAACACAGTC
O1940	<i>lacZ</i> forward	Amplify <i>lacZα</i> from pGEM5	GTTCTCTAGACTTATTAATAATTTATAGCTATTG
O1941	<i>lacZ</i> backward		CTTTATGCTTCCGGCTCGTATGTTG
			TCGTAACCGTGCATCTGCCAGTTTG





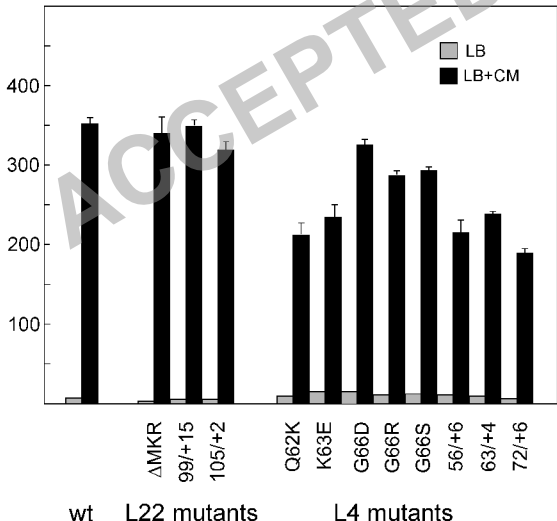
B.





B.

β -gal activity (M.U.)



A2450-A2451
(PTC)

A2058-A2059

K63

Q62

L4

S72

G56

G66

G91

L22

K98

R84

K83

M82

V105

