SsrA Tagging of Escherichia coli SecM at Its Translation Arrest Sequence

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SecM is expressed from the secM-secA operon and activates the expression of secA in response to secretion defects. The 3′-end of secM encodes an “arrest sequence,” which can interact with the ribosomal exit tunnel, preventing complete secM translation under secretion-defective conditions. In a cis-acting manner, ribosome stalling enhances secA translation. Pro166 is the last residue incorporated when SecM elongation is arrested. We report that secretion deficiencies lead to SsrA tagging of SecM after Pro166, Gly165, and likely Arg168. Northern blot analysis revealed the presence of a truncated secM transcript, likely issued from a secM-secA cleavage. The level of secM transcripts was decreased either when secM translation was totally prevented or when Pro166 was mutated. However, the accumulation of a truncated secM transcript required secM translation and was prevented when the SecM arrest sequence was inactivated by a point mutation changing Pro166 to Ala. We suggest that ribosome pausing at the site encoding the arrest sequence is required for formation of the truncated secM mRNA. SsrA tagging affected neither the presence of the secM mRNA nor secA expression, even under translation-defective conditions. It is therefore likely that SsrA tagging of SecM occurs only after cleavage of secM-secA mRNA within the secM open reading frame encoding the SecM arrest sequence. Accumulation of transcripts expressing arrested SecM generated growth inhibition that was alleviated by the SsrA tagging system. Therefore, SsrA tagging of SecM would rescue ribosomes to avoid excessive jamming of the translation apparatus on stopless secM mRNA.

Nascent proteins emerge from the 50 S ribosomal subunit through an exit tunnel with a length of ~100 Å and containing 35–40 amino acids of the elongating polypeptide. Its narrowness prevents peptide folding, and certain sequences from nascent peptides can interact with ribosomal elements of the tunnel to affect protein biosynthesis. The ribosome is therefore sensitive to the composition of the amino acid chain it is synthesizing, and the exit tunnel contributes to the dynamics of protein elongation (1). In Escherichia coli, this is exemplified by a sequence of the periplasmic protein SecM (secretion monitor) at a position close to its C terminus called the arrest sequence, which interacts with the ribosomal exit tunnel and causes elongation arrest within the ribosome (Refs. 2 and 3; for review, see Refs. 4 and 5). Ribosomal arrest is a key element in the regulation of SecA, an ATPase that targets protein precursors to the SecYEG core translocon for secretion. SecA is expressed from the secM-secA-mutT operon; its translation is up-regulated by a block in SecM secretion in a cis-specific manner (2, 6). A secondary structure encompassing the secA Shine-Dalgarno sequence of the secM-secA-mutT mRNA can conditionally inhibit secA translation. Due to its arrest sequence, secM translation stalls when its N-terminal signal sequence is not “pulled” by the protein export machinery (7, 8). Paused ribosomes are thought to mediate unfolding of the RNA secondary structure, thereby exposing the initiation signal for SecA biosynthesis (9). By this mechanism, the amount of SecA is adjusted to the intracellular demand for protein export (6).

Pausing of translating ribosomes can lead to protein tagging by the transfer-messenger RNA (tmRNA)1; also called SsrA in E. coli (for review, see Ref. 10). When charged with an alanine, tmRNA is recruited to A-sites of stalled ribosomes, and growing polypeptide chains are transferred to alanyl-tmRNA by a transpeptidation reaction. tmRNA then becomes a template for translation of an oligopeptide tag, which in E. coli corresponds to ANDENYALAA (11). The change of matrix from mRNA to tmRNA is referred to as trans-translation. This mechanism allows release of stalled ribosomes (11) and elimination of tagged products by specific proteases (12–15). Ribosome stalling and trans-translation occur during translation of truncated mRNAs lacking a stop codon because release factors are not recruited (11, 16–18). Such truncated messengers could result from mRNA damage, premature transcription termination, or degradation or cleavage by ribonucleases. The bacterial toxins RelE, MazF, and ChpB were recently shown to cleave mRNAs from mRNA damage, premature transcription termination, or degradation or cleavage by ribonucleases. The bacterial toxins RelE, MazF, and ChpB were recently shown to cleave mRNAs and induce to SsrA tagging (19–22). Cognate tRNA scarcity (23) and inefficient stop codons and/or specific sequences (24–28) generate ribosome pausing, which can also lead to SsrA tagging.

Here, we show that ribosomes stalled on the secM transcript are rescued by the SsrA tagging system. We show the presence of a truncated secM mRNA transcript likely issued from endonucleolytic cleavage of a secM-secA mRNA. It requires translation of secM mRNA and the integrity of the SecM arrest sequence. SsrA tagging does not influence secA expression but prevents the toxicity of accumulated non-exported SecM.

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1 The abbreviations used are: tmRNA, transfer-messenger RNA; CTABr, cetyltrimethylammonium bromide; CRP, cAMP receptor protein.
**TABLE I**

| Strain | Relevant genotypes and properties | Source/Ref. |
|--------|----------------------------------|-------------|
| C600   | thr-1 leuB6 hisA21 cyu-101 lacY1 glvV44(AS) lam-1 rfd1 glpR200 thi-1 | Laboratory collection |
| DH5αZ1 | ΔargF-lacI69 F80Δace258(M15) glvV44(AS) rfd1 gyrA96 nalR recA1 endA1 spoT1 thi-1 hsdR17 Z1(lacR tetR SpR) | 56 |
| 1512   | As W3110 strA::Km                  | 57 |
| A6     | From W3110 strA::Km                | 58 |
| MG1655 | Prototrophic                      | Laboratory collection |
| PhB2392| As MG1655 ssrAH7                   | See “Experimental Procedures” |
| PhB1907| As MG1655 ΔclpP-clpX               | 25 |
| PhB1805| As MG1655 ssrA::Km                 | 25 |
| PhB2162| As MG1655 ΔclpP-clpX ssrA::Km      | 25 |
| PhB1861| As MG1655 slyD::Km                 | 25 |
| PhB2431| As MG1655 slyD::Km ssrAH7          | 59 |
| MG1655Z1| Z1::lacR tetR SpR                   | 59 |
| PhB1745| As MG1655Z1 Δsp3::Km rpoB meta     | Laboratory collection |
| PhB2677| As MG1655Z1 ssrAH7                 | PhB2392 + P1/MG1655Z1 |
| PhB2497| As MG1655Z1 ΔsecA::Km              | PhB2497 + pCP20 |
| PhB2500| As MG1655Z1 ΔsecA                  | PhB2677 + P1/PhB1745 |
| PhB2693| As MG1655Z1 ssrAH7 Δsp3::Km        | PhB2392 + P1/MG1655Z1 |
| BW25113ΔhspB | ΔaraD-araB5′67 ΔlacZ4787 lacO-4000(lacP) sroS396(Am) rph-1 | 19 |
| BW25113ΔhspAK | ΔaraD-araB5′67 ΔlacZ4787 lacO-4000(lacP) sroS396(Am) rph-1 | 19 |
| BW25113ΔreiEBF | ΔaraD-araB5′67 ΔlacZ4787 lacO-4000(lacP) sroS396(Am) rph-1 | 19 |
| PhB2922| As MG1655Z1 ΔhspB:cat              | MG1655Z1 + P1/BW25113 ΔhspB |
| PhB2923| As MG1655Z1 ΔhspB:cat ΔhspB::aphA | MG1655Z1 + P1/BW25113 ΔhspB |
| PhB2924| As MG1655Z1 ΔreiEBF:aphA           | MG1655Z1 + P1/BW25113 ΔreiEBF |
| PhB2949| As MG1655Z1 ΔhspB                   | PhB2922 + pCP20 |
| PhB2950| As MG1655Z1 ΔhspB:cat ΔhspB::aphA  | PhB2923 + pCP20 |
| PhB2951| As MG1655Z1 ΔreiEBF:aphA           | PhB2924 + pCP20 |
| PhB2959| As MG1655Z1 ΔhspB:cat ΔhspB::aphA  | PhB2924 + pCP20 |
| PhB2962| As MG1655Z1 ΔreiEBF:aphA           | PhB2959 + P1/BW25113 ΔhspB |
| PR478  | ΔlacI169 araD139 rpsL150 thi B5301 deoC7 pts25 relA1 | 42 |
|        | secA::lacZ-f181 (ΔP9)              | |
| PhB2469| As PR478 ΔsrrA::Km                 | PR478 + P1/A6 |
| BL21(DE3)| ompT hsdS (rps-mc) gal dcm ΔDE3 (αimm21 lacI lacUV5 T7 RNA pol) | 60 |
| PhB3078| As BL21(DE3) ΔsrrA::Km             | BL21 + P1/A6 |

The pΔssmM2secM plasmid expresses a fusion protein between tandem FLAG epitopes and ΔssmM. The 5′-truncated secM gene was amplified from the chromosome using primers 5′-ATACCGGTCCG ACCTCCAGCA GCGCGCCGCA-A and 5′-CGGATCTCCAA TAAAATTCTCA AACGCC-3′. The PCR product was digested by Sall and BamHI and cloned into the Sall-BamHI sites of pZaJ1-M2N (25), giving rise to pΔssmM2secM.

The pΔssmM2secM plasmid expresses a fusion protein with internal tandem FLAG epitopes between Val325 and Asn56 of the SecM protein (with its signal sequence). It was obtained by cloning the linker resulting from the nucleotide annealing of 5′-GCTAGTTTA TCTATCTAT CTTTTAATTC TTATC CCCAT-3′ and 5′-ATG GATTAT AAGATGATG ATGAGATG ATG GATTATA AGAGATG ATGAGATG AGAGATG ATG -3′ into the PmI site of pΔssmM2secM.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Enzyme Assay**—The E. coli strains used in this work are presented in Table I. P1 vir-mediated transduction was carried out as described (29). Cells were grown at the temperatures indicated in Terrific broth, LB broth, or M63 broth supplemented with 0.4% glucose. Solid medium contained 15 mg/ml agar (29). Antibiotics were added as necessary at the following concentrations: ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; and kanamycin, 20 µg/ml. Expression from the Pn promoter was obtained by addition of 1 µM anhydrotetracycline. Expression of cloned inserts under the control of the T7 promoter in pET-3b derivatives (in BL21(DE3) pLys derivatives) was achieved by addition of isopropyl β-D-thiogalactopyranoside (1 mM) to the growth medium. Sodium azide was added where indicated to a final concentration of 0.02%. Bacterial growth was monitored at λabs, β-Galactosidase activity was determined as described (29).

**Plasmids and Molecular Biology Techniques**—Plasmid preparation, DNA cloning and ligation, PCR amplification, and DNA transformation were carried out according to standard protocols (30) or manufacturers’ instructions. DNA sequencing was performed by Eurogentec (Angers, France) or at the CNRS genomic platform (Gif-sur-Yvette, France). Oligonucleotides used for PCR amplification and for priming standard sequencing reactions were purchased from Sigma. E. coli strain C600 was the source of chromosomal DNA for PCR amplifications. Short descriptions of the plasmids used in this work are presented in Table II.

The secM gene was amplified using primers 5′-GGGGTTACCAT GTCCAGACCA GCGCGCCGCA-A and 5′-CGGATCTCCAA TAAAATTCTCA AACGCC-3′. The PCR product was digested with KpnI-BamHI and cloned into KpnI-BamHI-digested pZaJ1-M2N-C51 (31). The resulting plasmid, pΔssmM, expresses SecM precursor protein.

A truncated secM gene lacking its 5′-end was amplified using primers 5′-GGGGTTACCAT GTCCAGACCA GCGCGCCGCA-A and 5′-CGGATCTCCAA TAAAATTCTCA AACGCC-3′. The PCR product was digested with KpnI-BamHI and cloned into KpnI-BamHI-digested pZaJ1-M2N-C51 (31). The resulting plasmid, pΔssmM2secM, expresses the mature SecM protein lacking its signal sequence, ΔssmM SecM (Ser5-Ala106) (32).
TABLE II

| Plasmid | Relevant properties | Source/ref. |
|--------|---------------------|-------------|
| pCP20  | FLP recombinase-encoding vector | 61          |
| pSrAr1 | smpB srrA            | 25          |
| pSrAr1-H | smpB srrAH7 (SrrA-H) | 25          |
| pKO3   | Gene replacement vector | 33          |
| pKO3-ssrA-H | smpB srrAH7 (SrrA-H) | This study  |
| pZAS1-MCS1 | p13A origin vector | 31          |
| pSecM  | pZAS1-MCS1 derivative expressing SecM | This study  |
| pAssM | pZAS1-MCS1 derivative expressing truncated AssM protein with no signal sequence | This study  |
| pssM2SecM | pSecM derivative expressing ssM2SecM | This study  |
| pZAS1-M2N | pZAS1-MCS1 derivative vector | This study  |
| pAssM | pZAS1-M2N derivative expressing AssM2SecM | This study  |
| pMF11  | secM and secA-loc2 translational fusion | 62          |
| pLysS  | T7 lysozyme inhibits basal levels of T7 transcription in uninduced cell | Stratagene  |
| pET-3b | Expression system using T7 promoter | Stratagene  |
| pETSecM | pET-3b derivative expressing SecM under control of T7 promoter | This study  |
| pETSecM* | pET-3b derivative with secM initiation codon changed to stop codon; transcript under control of T7 promoter | This study  |
| pETAssM | pET-3b derivative expressing AssM under control of T7 promoter | This study  |
| pETAssM-P166A | pET-3b derivative expressing AssM with Pro166 changed to Ala under control of T7 promoter | This study  |
| pETSecM-P166A | pET-3b derivative with secM initiation codon changed to stop codon and corresponding Pro166 changed to Ala; transcript under control of T7 promoter | This study  |
| pETAssM-P166A | pET-3b derivative expressing AssM with Pro166 changed to Ala under control of T7 promoter | This study  |

Identification of SsrA-H7-tagged AssM by Mass Spectrometry—
Tryptic peptides from SsrA-H7-tagged AssM were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry according to a described protocol (25). Strain PhB2431 (with a slyD mutation to avoid sample contamination by the histidine-rich peptidyl cis,trans-isomerase SlyD) containing pAssM was used for identification of SsrA-H7-tagged AssM. The percentage of protein length covered by peptide matches was ~40% for SsrA-H7-tagged AssM.

Western Blotting—Following SDS-15% PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences) as described (36). Immunodetection was performed with anti-FLAG polyclonal antibody M2 and alkaline phosphatase-conjugated anti-rabbit antibodies (Sigma).

RESULTS

Elongation-arrested SecM Is SsrA-tagged—Elongation of secM translation is arrested when SecM is not translocated to...
FIG. 1. Elongation-arrested M2SecM derivatives are SsrA-tagged. M2SecM derivatives were analyzed by immunoblotting or immunoprecipitation with serum raised against the FLAG epitope (M2) in the wild-type strain (MG1655), the FLAG epitope (M2) in the wild-type strain expressing periplasmic ssM2SecM, and mature M2SecM derivatives, respectively. A, detection of ΔssM2secM gene products by immunoblotting with serum raised against the FLAG epitope (M2) in the wild-type strain (MG1655), ssrA7 (PhB2392), ΔclpP-clpX (PhB1907), and ssrA-Km ΔclpP-clpX (PhB2162) strains containing pΔssM2secM (+) or pΔ331-MCS1 (control vector; −) as indicated. B, detection of ssM2secM gene products by immunoblotting with serum raised against the FLAG epitope to allow immunodetection or immunoprecipitation of elongation-arrested SecM was achieved by preventing SecM export either (i) by using a signal sequence-less SecM derivative (ΔssM2secM) (Fig. 1A) or (ii) by impairing secretion via addition of sodium azide (0.02% final concentration) for 100 min (−) or left untreated (+) as indicated. C, CTABr fractionation of ΔssM2secM gene products in the wild-type strain (MG1655) and ssrA7 (PhB2392) strains containing pΔssM2secM. P and S indicate pellet and supernatant, respectively. Where indicated (−), samples were incubated with RNase A before fractionation. D, synthesis and stability of the ssM2secM gene products in a ΔsrrA Δpcre strain (PhB2693) containing pssM2secM. Cells were subjected to pulse-chase ([35S]Met) and immunoprecipitation analyses using anti-FLAG antibody M2. Where indicated (−), cultures were treated with sodium azide (0.02% final concentration) for 105 min. 

A

B

C

D

Elongation-arrested M2SecM derivatives are SsrA-tagged. M2SecM derivatives were analyzed by immunoblotting or immunoprecipitation with serum raised against the FLAG epitope (M2). T, A, and M indicate SsrA-tagged (by wild-type ssrA or ssrA7), elongation-arrested, and mature M2SecM derivatives, respectively. A, detection of ΔssM2secM gene products by immunoblotting with serum raised against the FLAG epitope (M2) in the wild-type strain (MG1655), ssrA7 (PhB2392), ΔclpP-clpX (PhB1907), and ssrA-Km ΔclpP-clpX (PhB2162) strains containing pΔssM2secM (+) or pΔ331-MCS1 (control vector; −) as indicated. B, detection of ssM2secM gene products by immunoblotting with serum raised against the FLAG epitope to allow immunodetection or immunoprecipitation of elongation-arrested SecM. Accumulation of elongation-arrested SecM was achieved by preventing SecM export either (i) by using a signal sequence-less SecM derivative (ΔssM2secM) (Fig. 1A) or (ii) by impairing secretion via addition of sodium azide (0.02% final concentration) for 100 min (−) or left untreated (+) as indicated. C, CTABr fractionation of ΔssM2secM gene products in the wild-type strain (MG1655) and ssrA7 (PhB2392) strains containing pΔssM2secM. P and S indicate pellet and supernatant, respectively. Where indicated (−), samples were incubated with RNase A before fractionation. D, synthesis and stability of the ssM2secM gene products in a ΔsrrA Δpcre strain (PhB2693) containing pssM2secM. Cells were subjected to pulse-chase ([35S]Met) and immunoprecipitation analyses using anti-FLAG antibody M2. Where indicated (−), cultures were treated with sodium azide (0.02% final concentration) for 105 min.

Cytoplasmic SsrA-tagged proteins are degraded by ClpP-dependent proteases. They can be stabilized and detected when ClpP is deficient (13–15). When ΔssM2secM was expressed in a ΔclpP-clpX background, a second translation product was detected (Fig. 1A, lane 4). This product was absent when ClpP-dependent proteases were active (Fig. 1A, lane 2) or when SsrA tagging was deficient (lane 5) and is likely the SsrA-tagged ΔssM2secM product. SsrA-tagged proteins can also be stabilized by means of a modified SsrA (SsrA-H7) encoding the peptide tag ANDENYHHHHH, which is not recognized by proteases (25). In an ΔsrrA7 background expressing ΔssM2secM, two translation products were detected using anti-FLAG antibody M2. The slower migrating protein of approximately 19 kDa (Fig. 1A, lane 3) corresponds to SsrA-H7-tagged ΔssM2secM.

In a wild-type strain expressing periplasmic ssM2secM, no translation product was detected by Western blotting using anti-FLAG antibody M2 (Fig. 1B, lane 2). It is likely that ssM2secM is efficiently synthesized and rapidly translocated to the periplasm, where the resulting mature M2secM protein is rapidly degraded by Tsp protease, as was reported for SecM (7). To confirm that ssM2secM behaves like native SecM, it was analyzed by immunoprecipitation using M2 antiserum after [35S]Met pulse-chase experiments in a Δpcre strain deficient for

the periplasm (7, 39). Since SsrA tagging of proteins can be induced by ribosome stalling, we hypothesized that elongation-arrested SecM protein is subject to SsrA tagging.

We constructed SecM derivatives containing a tandem FLAG epitope to allow immunodetection or immunoprecipitation of SecM. Expression of cytoplasmic ΔssM2secM gave rise to a protein of approximately 17 kDa, detected by Western blotting using anti-FLAG antibody M2 (Fig. 1A, lane 2). Bands often appeared as closely spaced doublets (see "Discussion"). SecM elongation-arrested peptidyl-tRNA was shown previously to transiently accumulate in wild-type cells, whereas the complete SecM protein is unstable (7), suggesting that the protein we detected (Fig. 1A, lane 2) corresponds to elongation-arrested ΔssM2secM. CTABr is used to precipitate peptidyl-tRNA molecules because of their RNA moiety (41). CTABr precipitation of total proteins from strains expressing ΔssM2secM led to the detection of a protein cross-reacting with antibody M2 (Fig. 1C, lanes 2 and 5). This band disappeared if the samples were treated by RNase prior to CTABr precipitation (Fig. 1C, lanes 3 and 6). These experiments demonstrate that the only detectable form of ΔssM2secM in the wild-type strain is an elongation-arrested peptidyl-tRNA (Fig. 1C, compared lanes 4–6).
SsrA Tagging of SecM

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**A**

| Plasmid | V | V | ΔssSecM |
|---------|---|---|---------|
| ssrA    | + | H7| H7      |

21 kDa → T

14 kDa → 1 2 3

**B**

SecM

F<sub>150</sub>STPVWSAQGIRAGPDF6QQR LT

SsrAH7-tagged SecM

F<sub>150</sub>STPVWSAQGIRAGPAANDENYHHHHHHHHHH

1979.83 Da

F<sub>150</sub>STPVWSAQGIRAGAANDENYHHHHHHHHHHHHHH

1883.78 Da

F<sub>150</sub>STPVWSAQGIRAAANDEMYHHHHHHHHHHHHHH HH

1755.72 Da

**Fig. 2.** AssSecM is SsrA-tagged at Pro<sup>166</sup>, Gly<sup>165</sup>, and likely Arg<sup>163</sup>. A, SDS-polyacrylamidegel of proteins affinity-purified on nicked-DBS, obtained by affinity chromatography on V<sub>9004</sub>/H9004 and lanes 1–3 are samples from strain PhB1851 (ssrA<sup>+</sup>) expressing pZA31-MCS1 (control vector), strain V<sub>9004</sub>/H9004 ssrA<sup>+</sup> ssr<sup>2</sup> , respectively. The two closely spaced bands indicated by the arrows were cut from the gel and analyzed separately by mass spectrometry. B, C-terminal amino acid sequences of SecM and SsrA-H7-tagged SecM products starting from the start codon. The C-terminal tryptic peptide from SsrA-H7-tagged ΔssSecM identified by mass spectrometry is shown in boldface (with experimental masses indicated below), and the SsrA-H7 tag is in italics.

Tsp protease (Fig. 1D). Two bands were detected: elongation-arrested ssM2SecM (slow migrating) and mature M2SecM (fast migrating). When secretion was efficient, elongation-arrested ssM2SecM was very unstable, probably because it matured rapidly and was exported to the periplasm. When secretion was impaired by addition of sodium azide to the growth medium, elongation-arrested ssM2SecM was stabilized, confirming that ssM2SecM was produced. The elongation-arrested ssM2SecM derivative was also detected when secretion was impaired by Western blotting using anti-FLAG antibody M2 (Fig. 1B, lane 6). In the presence of modified SsrA-H7 (stabilizing the tagged product), sodium azide addition to the growth medium resulted in the appearance of a second translation product of ~22 kDa (Fig. 1B, lane 6), corresponding to SsrA-H7-tagged ssM2SecM. Elongation-arrested ssM2SecM intensity was increased in the ΔssrA strain compared with the ssrA<sup>+</sup> strain (Fig. 1B, lanes 6 and 7), suggesting that the ΔsrrA mutation stabilizes elongation-arrested ssM2SecM.

Approximately half of the detected elongation-arrested M2SecM was tagged by the SsrA system (Fig. 1, A and B). We conclude that secM translation arrest allows its efficient SsrA tagging and subsequent degradation by ClpP-dependent proteases.

Trans-Translation of secM Occurs at Codons Expressing the Arrest Sequence—SsrA-H7 allows affinity purification of SsrA-H7-tagged proteins. To identify the SsrA tagging sites of SecM, we purified SsrA-H7-tagged proteins under conditions maximizing SecM elongation arrest. The non-exported ΔssSecM protein was overproduced (pΔssSecM) in an ssrA<sup>+</sup> strain. SsrA-H7-tagged proteins were affinity-purified and analyzed by gel electrophoresis. Two closely spaced bands at ~19 kDa were observed (Fig. 2A, lane 3) only in the ssrA<sup>+</sup> strain and in the presence of pΔssSecM. Proteins extracted from these bands were digested by trypsin, and the resulting peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Both bands gave the same results. Several peptide masses matched the mass of the SecM protein primary sequence, confirming that the observed bands correspond to SsrA-H<sub>7</sub>-tagged ΔssSecM protein. Two peptides had masses corresponding to those expected for junction peptides containing SecM residues 164–166 (AGP) or SecM residues 164 and 165 (AG) with the SsrA-H<sub>7</sub> tag and AANDENYH<sub>7</sub> (Fig. 2B). A third peptide had a mass corresponding to that of just the SsrA-H<sub>7</sub> tag, AANDENYH<sub>7</sub> (Fig. 2B). Tryptic digestion cleaved after lysine or arginine, indicating that the SsrA tagging of SecM occurs after one of these amino acids. These results show that SsrA tagging occurs after Gly<sup>165</sup> and Pro<sup>166</sup> and likely after Arg<sup>163</sup> of nascent SecM since all of the SsrA-tagged products analyzed have about the same total molecular masses.

*The ΔsrrA Mutation Does Not Affect secM Expression*—When SecM secretion is blocked, ribosomes pause on the secM coding sequence due to the SecM arrest sequence; this should cause unfolding of the secM-secA RNA secondary structure involved in secA repression, allowing secA translation initiation (9). Considering this model, SsrA tagging of elongation-arrested SecM could allow reformation of the inhibitory RNA secondary structure and thus reduce secA translation since the SsrA system can rescue stalled ribosomes. We therefore tested the effect of SsrA on SecA expression by means of a secA-lacZ translational fusion integrated at the secA locus (42). β-Galactosidase activities expressed from a secA-lacZ fusion in the ssrA<sup>+</sup> or ΔssrA strain were measured under growth conditions without sodium azide; no difference was observed (Fig. 3). This result was expected since secretion then occurs efficiently, and secM translation is subject only to a transient pause. When secretion was impaired by treating cells with sodium azide, a condition under which arrested SecM was subject to strong SsrA tagging (Fig. 1), SecA expression was greatly increased. (β-Galactosidase activity increased up to 8-fold after sodium azide was added to the medium.) However, induction was identical in the ssrA<sup>+</sup> and ΔssrA strains (Fig. 3). The presence or absence of ClpP-dependent proteases also had no effect on induction of secA-lacZ fusion expression in response to sodium azide addition to the culture medium (data not shown). We conclude that SsrA tagging and the subsequent degradation of elongation-arrested SecM do not influence SecA expression, even under very defective translational conditions in which SecM elongation arrest is prolonged and induces SsrA tagging of SecM.

Detection of secM and secM-secA mRNAs—It was recently demonstrated that ribosome stalling can cause mRNA cleavage at or next to arrest sites (43–47). We therefore considered that secM mRNA could be subject to such endoribonucleolytic cleavage. If so, it would result in a stop-less secM mRNA, which is the usual substrate for SsrA tagging. In that case, secM mRNA would be dissociated from secA-mutT RNA when tagging occurs, which is in keeping with the above result showing that tagging does not affect SecA expression.

To test this hypothesis, we performed Northern blot analyses on total RNAs from strains containing pET-3b derivatives carrying the secM gene and part of the secA gene. The pET-3b vector was chosen to obtain a high level of mRNA upon induction. We used several oligonucleotides probed for the secM, secM-secA intergenic, and secA RNA regions (Fig. 4A). With all plasmids and all probes, two large transcripts were detected (Fig. 4B): one of the size expected (~1.6 kilonucleotides) for the...
Northern blot analysis of strain ssrA

The open reading frames and positions of the probe, respectively. Shown is the induction of PR478 (x) caused by sodium azide addition. The secretion de- fect caused by sodium azide addition. PR478 (Δ and x) and its ssrA derivative (PhB2469, Δ and □) were grown at 37 °C in LB medium. Where indicated, sodium azide (0.02% final concentration) was added to cultures at $A_{600} = 0.03$, and the β-galactosidase activity (Miller units) was measured at various time points.

Results demonstrate that a truncated end of its coding sequence is produced together with the full-length secM-secA mRNA. No product of the expected size for secA mRNA only was detected using the intergenic or secA probe, suggesting that secA mRNA may be unstable if it is produced from a cleavage of secM-secA mRNA.

With pETSecM* (in which the secM initiation codon was mutagenized to a stop codon), we observed that the absence of secM translation drastically reduced the quantity of the full-length secM-secA mRNA (Fig. 4B, lane 2), probably because the transcript is not protected by translating ribosomes. We also detected a shorter product (>0.6 kilonucleotides) that interacted as well with the secM probe as with the intergenic probe, but not with the secA probe. These results demonstrate that a transcript including the complete secM coding sequence and all or part of the intergenic sequence between secM and secA is produced together with the full-length secM-secA mRNA. This transcript may be issued from a premature transcription termination. Interestingly, a mutation affecting nusB was previously isolated as a suppressor of a temperature-sensitive secA mutation (48), suggesting that altering anti-termination can stimulate secretion. However, no shorter transcript interacting only with the secM probe but not with the intergenic or secA probe could be detected. This result shows that preventing translation of the secM coding sequence prevents formation of the truncated secM transcript.

A mutation changing SecM Pro166 to Ala was shown previously to prevent translation arrest (2, 3). pETSecM, pETSecM*, and pETΔssSecM derivatives in which the secM sequence corresponding to the Pro166-encoding codon was mutated to express Ala were constructed, yielding pETSecM-P166A, pETSecM*-P166A, and pETΔssSecM-P166A, respectively. Upon Northern blot analysis using the same probes as described above, a similar transcript pattern as that obtained with the pETSecM* construct was observed at comparable quantities (Fig. 4B, lanes 5–7). This result shows that not only translation, but also the integrity of the SecM arrest sequence is needed for production of secM mRNA. The overall level of secM transcripts was decreased when the translation arrest-essential Pro166 was mutated to Ala166, as was observed when secM translation was prevented (Fig. 4B). In both cases, secA translation was decreased, and as a result, the absence of translating ribosomes could leave the mRNAs more susceptible to degradation.

All of Northern blot analyses described above were performed in either an ssrA+ or ssrA− background. The overall
indicated. Cultures were grown in the presence of anhydrotetracycline. Despite weak signals in the srrA− background, the transcript patterns were essentially the same as those in the srrA+ strain, showing that SsrA does not influence the production of the truncated secM transcript.

RelE, ChpB, and ChpAK and the SsrA System Are Not Required for the Production of Truncated secM mRNA—The bacterial toxins RelE, ChpB, and possibly ChpAK (MazF) cleave mRNAs in the ribosomal A-site (19–21) (although it was recently shown that ChpAK can cleave mRNAs independently of ribosomes (22)). We therefore considered that one of these bacterial toxins could be responsible for secM transcript cleavage in response to ribosome stalling. If so, toxin-defective mutants could prevent the accumulation of SsrA-tagged SecM. However, SecM was still observed in strains deleted for the relBEF, chpB, or chpAK gene (Fig. 5). As cleavage activities might be redundant, we also constructed a ΔrelBEF ΔchpB ΔchpAK strain. Again, no change in the level of tagging relative to the wild-type control was observed (Fig. 5). We therefore conclude that RelE, MazF, and ChpAK do not mediate mRNA cleavage. Our Northern blot experiments also showed that the SsrA tagging system is not needed for secM mRNA cleavage (Fig. 4). In addition, the ΔrelBEF ΔchpB ΔchpAK strain (data not shown) and ΔsrrA (Fig. 3) mutations do not affect the regulation of secA since no differences in secA-lacZ fusion expression were observed whether the secretion was blocked by sodium azide or not. We conclude that secM mRNA cleavage is probably not mediated by the bacterial toxins RelE, MazF, and ChpAK or by SsrA.

SsrA Counteracts Truncated secM mRNA Toxicity—We established above that SsrA is not required for secM mRNA cleavage. SsrA tagging nevertheless appears to have a biological role. Induction of ΔssecM transcription in the srrA+ or srrAH7 strain resulted in partial inhibition of cell growth, leading to the formation of small colonies compared with those obtained under non-induced conditions (Fig. 6) (7). When expression of ΔsSecM was induced, growth was more severely defective in the ΔsrrA strain than in the isogenic srrA+ strain. However, overproduction ofeither SsrA or SsrA-H7 (each of which mediates release of blocked ribosomes) alleviated the growth defect due to ΔsSecM expression (Fig. 6). ΔsSecM simulates conditions in which SecM secretion is blocked; the growth defect was not observed when wild-type (secreted) SecM was expressed (Fig. 6).

We initially considered that toxicity of ΔssecM expression could be due to overproduction of either the ΔsSecM protein or its coding mRNA. Our results show that the poor growth phenotype occurred regardless of whether the tagged ΔsSecM protein accumulated (in the presence of srrAH7) or was degraded (in the srrA− strain). We conclude that ribosome stalling on the mRNA encoding ΔsSecM, more than the protein itself, is responsible for toxicity. These results indicate that the SsrA tagging system alleviates toxicity by rescuing ribosomes that are stalled on truncated secM transcripts when SecM protein elongation is arrested and also by preventing accumulation of cleaved secM mRNA.

DISCUSSION

The sequence motif AR17 (FXXXXWXXXXGIRAGP, where X stands for any amino acid) present in SecM was shown to cause elongation arrest within the ribosome (2). The secretion-dependent SecA regulation is mediated by translation of secM and the presence of an mRNA stem-loop structure between secM and secA. All of the work presented here was performed on strains expressing constructs with secM and the complete secM-secA intergenic region. Work was performed simultaneously by Sunohara et al. (46) using a short portion of the secM gene encoding only the arrest sequence cloned between two reporter genes (crp and cer), resulting in the expression of CRP-GIRAGP-IIAGlc.

We present evidence that elongation-arrested SecM protein is SsrA-tagged after Pro166 Gly165, and probably Arg163. These sites correspond to three of the four SsrA tagging sites identified with the construct expressing the CRP-GIRAGP-IIAGlc hybrid (46). Both elongation-arrested SecM and SsrA-tagged SecM were detected as closely spaced doublets (Fig. 1), as also

**Fig. 5.** A SecM derivative is SsrA-H7-tagged in the absence of ChpB, ChpAK, and RelBEF bacterial toxins. ΔsSecM gene products were detected by immunoblotting with serum raised against the FLAG epitope (M2) in MG1655Z1 derivatives containing pΔssM2SecM or pSsrA-H7, as indicated (+). T and A indicate SsrA-tagged (by wild-type (WT) srrAH7) and elongation-arrested, respectively. The genotypes of derivatives carrying deletions of the ΔchpB (PhB2949), ΔchpAK (PhB2950), ΔrelBEF (PhB2951), and ΔchpB ΔchpAK ΔrelBEF (PhB2962) genes are indicated. Cultures were grown in the presence of anhydrotetracycline.

**Fig. 6.** The SsrA/SmpB system counteracts ΔsSecM toxicity. Cultures of strains MG1655Z1 (srrA+), PhB2500 (ΔsrrA), and PhB2677 (srrAH7) carrying pSsrA1, pSsrA-H7, control vector pZA31-MCS1, pSecM, and/or pΔssSecM were grown overnight in LB medium at 37 °C. Serial 10-fold dilutions (from left to right) were spotted (5 μl) onto M63 plates with (left panels) or without (right panels; control experiment) anhydrotetracycline (aTc), the inducer of secM and ΔssSecM transcription.

+ aTc - aTc

ssrAΔ

ΔssrA

ΔssrA/H7

pSsrA1

pSSrA-H7

ΔssSecM

pSecM

pSsrA1

pSSrA-H7

ΔssSecM

pSecM

pSsrA1

pSSrA-H7

pΔssSecM

pSSrA1

pSSrA-H7

ΔssSecM

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA

ΔssrA/H7

ΔssrA
reported previously (46). In the case of SsrA-H$_7$-tagged SecM (Fig. 2), the two closely spaced bands separately analyzed by mass spectrometry showed the presence of similar peptide species, probably due to cross-contamination. One band should correspond to SsrA tagging of ΔssSecM at Pro\textsuperscript{166} and Gly\textsuperscript{165} whereas the other band may correspond to tagging at Arg\textsuperscript{163}.

Our Northern blot results show that a truncated secM mRNA was produced together with the full-length secM-secA mRNA. In principle, it could arise by premature transcription termination, by cleavage of the secM mRNA before translation, or by cleavage when the ribosome pauses when synthesizing the arrest sequence. Yet, we have shown that formation of the truncated secM mRNA depends on secM translation and possibly on the integrity of the ribosome pausing site in secM mRNA (Fig. 4), supporting a cotranslational cleavage mechanism. Using the construct expressing CRP-GIRAGP-IIA\textsuperscript{Glc}, Sunohara et al. (46) observed five clusters of mRNA cleavage sites within their hybrid open reading frame, only one which is within the sequence encoding GIRAGP. We also observed cleavage sites at codons expressing the arrest sequence of natural SecM. We therefore propose that SsrA tagging of natural SecM occurs due to secM mRNA cleavage at or around the Pro\textsuperscript{166}-encoding sequence, leading to the appearance of a stop-less secM mRNA, which is the usual substrate for trans-translation.

Truncated secM mRNA production occurs independently of SsrA tagging. Accumulation of secM transcripts encoding non-exported SecM generates a growth defect, which is alleviated by SsrA tagging activity (Fig. 6). We propose that one role of the SsrA tagging system is to clear ribosomes stalled on truncated secM messengers. This activity may serve to avoid excessive jamming of the translation apparatus when translation is impaired. Furthermore, the SsrA tagging system facilitates degradation of messengers lacking a stop codon (49), so it is likely that SsrA tagging of SecM may also prevent accumulation of truncated secM transcripts.

SsrA Tagging Rescues Ribosomes Stalled on Stop-less secM mRNA—The SsrA tagging system can operate on idle ribosomes at the 3'-end of the stop-less truncated secM mRNA and perhaps on ribosomes stalled on the uncleaved secM-secA-mutT RNA. In the latter case, ribosomes released by the SsrA system would stabilize the secA regulatory structure and thereby limit secA expression. If SsrA tagging occurs on truncated secM mRNA, which is dissociated from secA mRNA, secA translation should not be affected by the presence of SsrA. Despite its involvement in rescuing SecM stalled ribosomes, the integrity of the SsrA tagging system is not required for regulated expression of SecA (ΔssrA) (Fig. 3) or ΔsmpB (data not shown). SsrA tagging of SecM is likely a consequence of secM-secA-mutT RNA cleavage at the end of the secM coding sequence. In E. coli, ribosome stalling seems to be a prerequisite for SsrA recruitment to the ribosome. It is therefore likely that the ribosomal A-site is always empty prior to SsrA tagging either because the mRNA was initially stop-less or because it became so after degradation or after ribosome-associated endoribonucleolytic cleavage (44, 46).

Like the SecM arrest sequence, erythromycin causes elongation arrest by interacting with the exit tunnel (50–52). Two mutations (rrlB2058 and rplV281) confer erythromycin resistance; these mutations also bypass elongation arrest during secM translation (2), thus revealing mechanistic similarities between the effects of erythromycin and SecM elongation arrest. Furthermore, SsrA-defective cells are more sensitive to erythromycin compared with wild-type E. coli cells (17) as well as to accumulation of elongation-arrested SecM. This suggests that, in both cases, stalled ribosomes can be rescued by the SsrA system. The recruitment of the SsrA system in the A-site of the ribosome may induce a conformational change that releases the interaction of elongation-arrested proteins with the exit tunnel of the ribosome so that trans-translation can occur. Alternatively, secM mRNA cleavage releases the interaction of nascent SecM with the ribosome. SsrA contributes to translational quality control by avoiding the accumulation of stalled ribosomes.

An Endoribonucleolytic Activity within the Ribosome?—It was proposed that the exit tunnel interacts with nascent SecM, leading to inhibition of peptidyl transfer and/or of the ribosomal translocation reaction. Once arrested, the peptidyl-tRNA molecule should be located either in the P- or A-site of the ribosome. As the last amino acid added to nascent SecM when translation elongation stalls is Pro\textsuperscript{166}, its encoding codon is predictably located within the ribosome either in the P- or A-site. This positioning suggests that the secM-secA-mutT RNA cleavage is likely to occur at the Pro\textsuperscript{166}-encoding site within the ribosome (46). Since SsrA tagging of SecM occurs as efficiently in strains defective for bacterial toxins as in a wild-type strain, we conclude that ChpB, ChpAK, and RelBEF are not involved in the secM-secA-mutT RNA cleavage (Fig. 5). Furthermore, the cleavage of mRNA encoding CRP-GIRAGP-IIA\textsuperscript{Glc} still occurs in cells lacking RNase III, RNase E, RNase G, and RelE (46). Recently, ribosomes pausing at stop or sense codons were shown to induce translation-dependent cleavages of their transcripts within or immediately adjacent to the ribosomal A-site (44, 46, 53). Our data show that the appearance of a stop-less secM mRNA requires translation of secM and the integrity of the SecM arrest sequence (Fig. 4B). These overall results suggest the existence of a ribosome-associated endoribonucleolytic activity. Ribosome-mediated mRNA cleavage could participate in a general quality control of protein synthesis when translation elongation or translation termination is affected. Cleavage would induce SsrA tagging and degradation of proteins blocked within ribosomes to allow release and recycling of ribosomes.

secM mRNA Cleavage and SecM-mediated SecA Regulation?—secM-secA-mutT RNA forms a stem-loop structure encompassing the 3'-end of secM and the secA Shine-Dalgarno sequence (54). Mutations that partly disrupt the base of the helix structure were shown to cause severe defects in secA repression (55). The two last nucleotides of the CCU codon encoding Pro\textsuperscript{166} of SecM are predicted to pair with and thereby occlude the secA Shine-Dalgarno sequence and repress secA translation. It was proposed that SecM-mediated SecA regulation depends on secM translation arrest; the paused ribosome unfolds the stem-loop structure, rendering the secA Shine-Dalgarno sequence accessible for translation (7). A consequence of secM mRNA cleavage at the Pro\textsuperscript{166}-encoding codon (compatible with the observed SsrA tagging of SecM after Gly\textsuperscript{165}) would be to partly expose the secA Shine-Dalgarno sequence on the downstream cleavage product, which could promote initiation of secA translation. We observed SsrA tagging of SecM when secretion was defective. This suggests that such conditions promote not only translation arrest, but maybe also secM mRNA cleavage. One possibility is that ribosome stalling due to the SecM arrest sequence induces secM-secA-mutT RNA cleavage within the ribosome, which may release a translation-competent secA mRNA, if it exists. Expression of SecA in response to secretion defects would be a consequence of mRNA cleavage induced by an arrested ribosome. We did not detect an mRNA encoding solely part of SecA; this may reflect its extreme instability or the absence of a complete secA transcript. An alternative is that secM mRNA cleavage may occur
only on a shorter transcript that does not include secA but includes the secM-secA intergenic sequence. In that case, secM mRNA processing and subsequent SarA tagging of SecM would not be associated with the secretion-responsive regulation of secM. Identification of the ribosome-dependent endonucleolytic activity involved in this process will be instrumental in determining the role of secM mRNA cleavage in the secretion-responsive regulation of secA.

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