A ribosome–nascent chain sensor of membrane protein biogenesis in *Bacillus subtilis*

Shinobu Chiba¹, Anne Lamsa and Kit Pogliano*

Division of Biological Sciences, University of California, San Diego, CA, USA

Proteins in the YidC/Oxa1/Alb3 family have essential functions in membrane protein insertion and folding. *Bacillus subtilis* encodes two YidC homologs, one that is constitutively expressed (*spoIIIJ/yidC1*) and a second (*yqjG/yidC2*) that is induced in *spoIIIJ* mutants. Regulated induction of *yidC2* allows *B. subtilis* to maintain capacity of the membrane protein insertion pathway. We here show that a gene located upstream of *yidC2* (*mifM/yqzJ*) serves as a sensor of SpoIIIJ activity that regulates *yidC2* translation. Decreased SpoIIIJ levels or deletion of the MifM transmembrane domain arrests *mifM* translation and unfolds an mRNA hairpin that otherwise blocks initiation of *yidC2* translation. This regulated translational arrest and *yidC2* induction require a specific interaction between the MifM C-terminus and the ribosomal polypeptide exit tunnel. MifM therefore acts as a ribosome–nascent chain complex rather than as a fully synthesized protein. *B. subtilis* MifM and the previously described secretion monitor SecM in *Escherichia coli* thereby provide examples of the parallel evolution of two regulatory nascent chains that monitor different protein export pathways by a shared molecular mechanism.

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Introduction

Approximately 30% of cellular proteins are translocated across or inserted into the membrane using a conserved and essential membrane translocation apparatus. In the Eubacteria, movement of proteins across the membrane depends on three integral membrane proteins that assemble a protein-conducting channel, SecY and SecE, which are present in all cells, and SecG, which is replaced by Sec61 β in eukaryotes and archaea (reviewed by Pohlschroder *et al*,

2005; Papanikou *et al*, 2007; Rapoport, 2007; Driessen and Nouwen, 2008). Bacterial protein secretion depends on the SecA ATPase, which functions as a motor to translocate secreted proteins through the SecYEG channel. The insertion of integral membrane proteins into the membrane is also essential and the machinery for this process partially overlaps with the secretory apparatus: most substrates seem to require the SecYEG protein-conducting channel, but only substrates with large extracellular domains require the SecA ATPase for membrane insertion (Kol *et al*, 2008; Xie and Dalbey, 2008). The membrane insertion of many proteins also requires YidC, a conserved membrane protein in the YidC/Oxa1/Alb3 family that is required for membrane protein insertion and assembly of membrane protein complexes in bacteria, mitochondria and chloroplasts (Yi and Dalbey, 2005; van der Laan *et al*, 2005; Kiefer and Kuhn, 2007). Biochemical experiments suggest that some membrane proteins first interact with the SecYEG channel and are later transferred to YidC for integration into the lipid bilayer or for folding into functional state (Urbanus *et al*, 2001; van der Laan *et al*, 2001). However, certain membrane proteins that lack large extracellular domains are inserted into the membrane in a manner that depends on YidC but not on SecYEG, suggesting flexibility in the pathway for membrane protein biogenesis (Kiefer and Kuhn, 2007; Xie and Dalbey, 2008). The cryoelectron microscopy images of YidC (Lotz *et al*, 2008; Kohler *et al*, 2009) and the ability of purified YidC to insert certain proteins into the membrane in the absence of the Sec proteins (Serek *et al*, 2004; van der Laan *et al*, 2004), suggest that YidC provides a second protein-conducting channel.

The essentiality of protein secretion and membrane protein biogenesis makes it likely that all cells can respond to decreased secretion capacity by either prioritizing certain substrates, as in eukaryotes (Kang *et al*, 2006; Oyadomari *et al*, 2006; Hegde and Kang, 2008), or by increasing the secretion capacity of the cell. Indeed, *Escherichia coli* and closely related bacteria have a feedback mechanism to maintain cellular protein secretion capacity by increasing SecA translation when the SecYEG pathway is compromised (Oliver and Beckwith, 1982; Schmidt and Oliver, 1989). This feedback mechanism depends on a gene located upstream of *secA*, *secM*, that acts as a ribosome–nascent chain complex to monitor protein secretion capacity (McNicholas *et al*, 1997; Schmidt *et al*, 1988; Nakatogawa and Ito, 2001, 2002). Prior data suggested that *Bacillus subtilis* cells might have the ability to monitor the YidC-dependent membrane protein insertion pathway (Rubio *et al*, 2005). Specifically, *B. subtilis*, like many other Gram-positive bacteria, encodes two YidC homologs (SpoIIIJ and YqjG) either of which can support viability (Murakami *et al*, 2002; Tjalsma *et al*, 2003). However, the role of these proteins in *B. subtilis* remains unclear (Murakami *et al*, 2002; Tjalsma *et al*, 2003), in contrast those encoded by *Streptococcus mutans*, which clearly has a role in membrane protein insertion

*Corresponding author. Division of Biological Sciences, University of California, San Diego, 9500 Gilman Drive La Jolla, San Diego, CA 92093-0377, USA. Tel.: +1 858 822 1314;

Fax: +1 858 822 5740; E-mail: kpogliano@ucsd.edu

¹Present address: Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

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(Hasona *et al*, 2005; Dong *et al*, 2008; Funes *et al*, 2009). The *B. subtilis* *spoIIJ* gene is likely the primary *yidC* homolog (YidC1), because it is constitutively expressed and located in a similar chromosomal context as *E. coli* *yidC* (Errington *et al*, 1992). The second *yidC* homolog (*yqjG*; here renamed *yidC2*) is likely the backup system for *spoIIJ* (*yidC1*), because it is induced in *spoIIJ* mutants (Rubio *et al*, 2005). This latter observation suggests that *B. subtilis* has a mechanism by which it can sense changing SpoIIJ activity and increase YidC2 levels as needed to maintain appropriate cellular levels of YidC activity.

We here describe the mechanism by which *B. subtilis* monitors SpoIIJ (YidC1) activity. Specifically, we show that YidC2 induction involves the product of the upstream gene, *yqzJ*, which is in an operon with *yidC2*, and an mRNA hairpin that occludes the *yidC2* Shine–Dalgarno (SD) site. The *yqzJ* gene encodes a small membrane protein with an N-terminal transmembrane (TM) segment that is a likely substrate of SpoIIJ (Xie and Dalbey, 2008). YqzJ also has a C-terminal translational arrest domain that interacts with the ribosomal polypeptide exit tunnel to induce a stable translational arrest when SpoIIJ is absent or limiting. This translational arrest positions the stalled ribosome over the 5' region of the mRNA hairpin, thereby exposing the *yidC2* SD site and allowing increased translational initiation. Membrane insertion relieves the translational arrest, so when membrane protein insertion is inhibited, the hairpin remains unfolded for longer periods of time, allowing additional YidC2 to be synthesized until cellular membrane protein insertion capacity is restored. Thus, YqzJ acts as a cellular monitor of SpoIIJ-dependent membrane protein insertion while it is a ribosome–nascent chain complex, rather than as a fully synthesized protein.

The regulatory mechanism we describe bears striking similarities to the *E. coli* protein secretion monitor SecM. We therefore propose renaming *yqzJ* as *mifM*, for membrane protein insertion and folding monitor. However, genomic and sequence comparisons between MifM and SecM reveal that the translational arrest motifs of these two regulatory proteins share no detectable sequence similarity and that they are found in different bacterial kingdoms. This suggests that SecM and MifM are independently evolved ribosome–nascent chain sensors that use a common molecular mechanism to monitor different protein export pathways.

Results

Disruption of *spoIIJ* upregulates *yidC2* expression at the translational level

To understand the mechanism of *yidC2* (*yqjG*) induction in *spoIIJ* mutants, we first investigated whether regulation occurs at the level of transcription, translation or protein stability. The *yidC2* gene is in an operon with the ORF *yqzJ* (here renamed *mifM*, as described below), which encodes a 95 amino acid protein (Figure 1A). As shown earlier (Rubio *et al*, 2005), an in-frame fusion of *lacZ* to the stop codon of *yidC2* (*yidC2*²⁷⁵–*lacZ*; Figure 1Aa) exhibits elevated β -galactosidase activity in *spoIIJ* strains (Figure 1Ba). This was also the case for an in-frame fusion of *lacZ* after the 6th codon of *yidC2* (*yidC2*⁶–*lacZ*; Figure 1Ab and Bb), so it is unlikely that YidC2 induction is due to increased proteolytic stability of YidC2 in the absence of SpoIIJ. If *yidC2* induction occurs by

modulating *mifM*–*yidC2* transcription or *mifM* translational initiation, then an in-frame fusion of *lacZ* after the 5th codon of *mifM* (*mifM*⁵–*lacZ*; Figure 1Ac) should also show elevated β -galactosidase activity in *spoIIJ* strains. However, identical levels of β -galactosidase activity were observed in the wild-type and *spoIIJ* strains with the *mifM*⁵–*lacZ* fusion (Figure 1Bc). Thus, the absence of SpoIIJ is likely to induce *yidC2* translation in a manner independent of *mifM* transcription and translational initiation.

mifM translation disrupts a stem loop that blocks *yidC2* translation

To further understand the mechanism by which *yidC2* is regulated, we performed a genetic screen to isolate mutants that induce *yidC2*. A strain containing the *yidC2*⁶–*lacZ* fusion was mutagenized with NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) and plated on DSM plates containing the β -galactosidase indicator XG. Blue, Lac⁺ colonies were picked and purified. Of the 150 Lac⁺ mutants, 37 contained mutations in *spoIIJ* and 17 were 100% linked to the *yidC2*⁶–*lacZ* reported gene. DNA sequence analysis revealed that eight of the *spoIIJ* mutations altered the SD sequence (Supplementary data), suggesting that reducing the level SpoIIJ protein is sufficient to induce *yidC2*. All of the mutations linked to *yidC*⁶–*lacZ* were within the upstream ORF, *mifM*. Ten *mifM* mutants had the same point mutation, a G–A transition at nucleotide 16, causing a glutamate to lysine substitution at the 6th amino acid of MifM; hereafter referred to as the class I mutation. The remaining seven mutations were at three sites near the 3' end of *mifM*, C275T (Ser92Thr), C277T (Leu93Phe) or C280T (Leu94Phe); hereafter referred to as the class II mutations. These results suggested that *mifM* has an important function in regulation of *yidC2* expression.

Examination of the 5' end of *yidC2* revealed a potential stem loop that could mask the *yidC2* SD site (Figure 1C). The 5' half of the stem loop is located within the 3' end of *mifM*, where the class II mutations are located (Figure 1C). We therefore hypothesized that the stem loop repressed the initiation of *yidC2* translation. In keeping with this idea, all class II mutations disrupt G–C pairing of the stem loop, suggesting that the mutations destabilize the structure, thereby leading to high-level constitutive expression of *yidC2*. To test this model, we introduced silent mutations that disrupt the structure without changing the MifM amino acid sequence (*yidC2*⁶–*lacZ*(Δ stem); Figure 1Df). Disrupting the stem loop indeed caused high-level constitutive expression of *yidC2* in wild-type and *spoIIJ* strains (Figure 1E), suggesting that the stem loop inhibits *yidC2* translation. To test whether *mifM* translation is necessary for *yidC2* translation, we first terminated translation before the ribosome reached the stem loop by introducing a TAA stop codon at codon 86 (Figure 1Dg). This abolished *yidC2* induction in a *spoIIJ* strain and slightly reduced *yidC2* expression in wild type (Figure 1Fg). We next duplicated six *mifM* codons (91–96) including the stop codon, so that wild-type MifM was synthesized but translation stopped before the ribosome moved into the stem loop (Figure 1Dh). This also resulted in low and constitutive expression of *yidC2* (Figure 1Fh) that was increased when the stem loop was disrupted (not shown). We conclude that translation of *mifM* into the 5' half of the stem loop is necessary for high-level *yidC2* expression.

Figure 1 *mifM* regulates *yidC2* expression. (A) Genetic context of *yqjG* (*yidC2*) and in frame translational *lacZ* fusions to the 3′-end of *yidC2* (a) or *mifM* (e), producing *YidC2*²⁷⁵–*LacZ* and *MifM*⁹⁵–*LacZ*, respectively, and after *yidC2* codon 6 (b, d) and *mifM* codon 5 (c). Construct (d) also contains an in-frame deletion of the *mifM* region encoding the transmembrane (TM) segment. (B) β-galactosidase (β-gal) activity in wild type (white) or the *spoIIJ* mutant (black). The fusions (a–e) correspond to those shown in (A). (C) Predicted secondary structure of *mifM*–*yidC2* mRNA. SD, Shine–Dalgarno site recognized by the ribosome to initiate translation. The class II mutations of *mifM* are indicated by arrows with residue’s numbers. (D) Diagram of *mifM* mutants in the *yidC2*⁶–*lacZ* fusion. (f) Silent mutations to disrupt the hairpin without changing *MifM* amino acids (*yidC2*⁶–*lacZ*(Δstem)). (g) Stop codon at codon 86. (h) Duplication of codons 91–96 (including the stop codon) to allow translation of full-length *mifM* without unfolding the hairpin. (E) β-gal activity with the stem-loop mutation shown in (D) (f). (F) Translation through the hairpin is required for *yidC2* expression. β-gal activity of strain with mutations (g) and (h) from (D). wt, wild type. (G–H) *mifM* functions *in cis*. Expression of *yidC2*–GFP monitored by fluorescence microscopy with identical GFP (green) exposures and image adjustments for each strain; membranes were stained with FM 4-64 (red). (G) Expression of *yidC2*–GFP downstream of wild-type *mifM* in wild type (H) Expression of *yidC2*–GFP downstream of wild-type *mifM* in *spoIIJ* (I) *MifM*ΔTM expressed in a wild-type strain with *yidC2*–gfp downstream of wild-type *mifM*.

MifM senses membrane protein insertion

SpoIIJ and YidC2 are likely involved in membrane protein biogenesis or protein secretion (Tjalsma *et al*, 2003; Camp and Losick, 2008). Interestingly, *MifM* is predicted to be a small membrane protein with a single N-terminal TM segment (amino acids 12–34) and a C-terminal cytoplasmic domain (amino acids 35–95). This topology is similar to that of *E. coli* proteins that are inserted into the membrane in a manner dependent on the SpoIIJ homologue YidC

(Xie and Dalbey, 2008), suggesting that *MifM* is inserted into the membrane by SpoIIJ. Interestingly, the class I *MifM* mutation changes the charge of the N-terminal domain from negative to positive, which might produce a membrane insertion defect that induces *yidC2*, thereby mimicking SpoIIJ limitation. To test this model, we deleted the region encoding the *MifM* TM segment and found that it caused elevated *yidC2* expression in wild-type cells (Figure 1Ad and Bd). These results suggest that *MifM* serves as a sensor of

SpoIIJ activity that upregulates *yidC2* when SpoIIJ activity is limiting and membrane insertion is blocked.

MifM is a cis-acting regulator of *yidC2* translation

If *mifM* translation unfolds the stem loop in a manner regulated by SpoIIJ-dependent membrane insertion of MifM, then it should regulate YidC2 expression only *in cis*. To test this prediction, we expressed MifM(Δ TM) *in trans* to *yidC2-gfp* with wild-type *mifM* upstream. The strain carrying only *yidC2-gfp* showed weak GFP fluorescence in wild-type cells (Figure 1G), and stronger membrane-associated GFP fluorescence in the *spoIIJ* background (Figure 1H) as expected (Rubio *et al*, 2005). Expression of MifM(Δ TM) did not elevate the YidC2-GFP signal (Figure 1I) unless it was encoded upstream of YidC2-GFP, indicating that *mifM* functions only *in cis*. Together these results suggest that MifM is a *cis*-acting regulator of *yidC2* translation that senses SpoIIJ activity, likely when it is inserted into the membrane as a ribosome-associated nascent chain. We therefore renamed its gene (formerly *yqzJ*) *mifM* for membrane insertion and folding monitor.

MifM is incompletely translated in the absence of SpoIIJ

The above results suggest that in the absence of SpoIIJ, the *mifM-yidC2* stem loop is more efficiently unfolded than in the presence of SpoIIJ. Unfolding could be accomplished either by allowing more ribosomes to translate *mifM-yidC2* mRNA or by having ribosomes move more slowly across the stem loop so it remains unfolded for longer times. The former model is unlikely because the initiation of *mifM* translation occurs at similar levels in the presence or absence of SpoIIJ (Figure 1Ac and Bc). We therefore hypothesized that the absence of SpoIIJ might stall the ribosome in a location that occluded the 5' side of the stem loop thereby exposing the *yidC2* SD. To test this idea, we fused *lacZ* at the 3'-end of the *mifM* coding sequence (producing *mifM*⁹⁵-*lacZ*; Figure 1Ae). This fusion showed lower β -galactosidase activity when *spoIIJ* was disrupted, as expected if *mifM* translation was attenuated under these conditions (Figure 1Be). This suggests that *mifM* translation is arrested when membrane insertion of MifM is blocked by the *spoIIJ* mutation or deletion of the MifM TM domain.

Detection of a MifM translational intermediate

If *mifM* translation is arrested in the absence of membrane insertion, it might be possible to observe a translational intermediate in *mifM*(Δ TM) strains. We therefore constructed strains expressing an N-terminal fusion of GFP to MifM lacking its N-terminal TM domain with or without a C-terminal FLAG-tag for detection of the full-length protein (Figure 2A; GFP-MifM³⁵⁻⁹⁵-FLAG and GFP-MifM³⁵⁻⁹⁵, respectively). Immunoblotting using GFP-specific antibodies detected two major products in the strain expressing GFP-MifM³⁵⁻⁹⁵ (Figure 2B, lane 2) and an additional larger minor product in the strain expressing GFP-MifM³⁵⁻⁹⁵-FLAG (Figure 2B, lane 3). This minor product (band a) was detected with both GFP- and FLAG-specific antibodies (Figure 2B, lanes 3 and 6), so it must be full-length GFP-MifM³⁵⁻⁹⁵-FLAG. However, the shorter products (bands b and c) were not detected by FLAG-specific antibodies, so they must be N-terminal fragments (Figure 2A). To test whether these

Figure 2 Visualization of MifM translational arrest. (A) The C-terminus of GFP was fused to amino acids 35–95 of MifM with or without the C-terminal FLAG-tag. (a–c) Products visualized in immunoblots. (B) Immunoblotting of GFP–MifM^{35–95} derivatives. Proteins from strains expressing GFP–MifM^{35–95} (lanes 2, 5), GFP–MifM^{35–95}-FLAG (3, 6) or no GFP fusion (1, 4) analysed by immunoblotting with anti-GFP (left panel) or anti-FLAG (right panel). (C) CTABr fractionation of GFP–MifM^{35–95}. Proteins from strains expressing GFP–MifM^{35–95} (lanes 2, 5, 8, 11, 14), GFP–MifM^{35–95}-FLAG (3, 6, 9, 12, 15) or no GFP fusion (1, 4, 7, 10, 13) analysed by immunoblotting with anti-GFP after CTABr precipitation. Samples 10–15 were treated with RNaseA before CTABr. WCL, whole cell lysate; ppt, precipitated fraction; sup, supernatant.

shorter products were translational intermediates or degradation products, we used CTABr precipitation (Gilmore *et al*, 1991; Nakatogawa and Ito, 2001), which precipitates RNA and RNA–protein complexes such as translational intermediates that retain a covalently attached tRNA at the C-terminus. Fragment b but not fragment c was precipitated by CTABr (Figure 2C) and this CTABr precipitation was abolished by RNase treatment. Thus, fragment b is an MifM translational intermediate whereas fragment c is likely a degradation product. These results show that *mifM*(Δ TM) undergoes a strong translational arrest such that the arrest species appears more abundant than full-length protein. The apparent size of fragment b suggests that the elongation arrest occurs within the 3'-region of *mifM*, a position at which the arrested ribosome could prevent formation of the mRNA hairpin that blocks *yidC2* translation.

A specific amino acid sequence is required for MifM translational arrest

To further elucidate the mechanism by which *mifM* regulates *yidC2*, we tested the relative importance of the *mifM* mRNA and amino acid sequences in the region of the hairpin. To do so, we designed a frame-shift mutation within *mifM* that would produce an mRNA sequence almost identical to native *mifM* but encode a completely different amino acid sequence. We first introduced an extra stop codon and a silent mutation (Figure 3Aa) to prevent out of frame translation from producing a product much longer than native MifM, and to prevent premature translational termination, respectively. The *yidC2*⁶-*lacZ* fusion with these two mutations showed low-level expression in the wild-type strain and induction in the *spoIIJ* strain to levels similar to the wild-type fusion

Figure 3 Identification of MifM amino acids required for translational arrest. (A) The *mifM* frame-shift mutant; white characters in black box indicate the mutations. Arrows show site of stem loop. (B) Introduction of stop codons in *mifM* (*). The number at left represents the codon substituted by a TAA stop codon. (C) β -gal activity of mutants in A in wild type (white) or *spoIIIJ* (black). (D) β -gal activity of mutants in B in wild type (white) or *spoIIIJ* (black). (E) Ala-replacement of GFP–MifM^{35–95}–FLAG at the *mifM* codons indicated by numbers. Immunoblotting with anti-GFP and anti-FLAG. Arrest-defective substitutions accumulate full-length protein. (F) β -gal activity of *yidC2*⁶–*lacZ* containing the indicated MifM Ala-replacements, in wild type (white) or *spoIIIJ* (black).

(Figure 3Ca). When then introduced a frame-shift mutation at codon 78 (Figure 3Ab), which resulted in low and constitutive *yidC2*⁶–*lacZ* expression (Figure 3Cb), showing that the

translational arrest was abolished. We conclude that a specific amino acid sequence at the MifM C-terminus is required for translational arrest.

Identification of the site of translational arrest

Stop codons before the translational arrest site should prematurely release the ribosome from the mRNA, allowing the stem loop to refold and inhibiting YidC2 expression, whereas those after the arrest site should have no effect. We therefore introduced stop codons immediately to the 5' of the stem loop to determine whether the mutation prevented *yidC2* induction in the *spoIIJ* strain (Figure 3B). Stop codons before codon 88 blocked *yidC2* induction (Figure 3D), whereas a stop codon at codon 90 showed wild-type induction (Figure 3D). These results indicate that *mifM* translation must continue to codon 88 to allow *yidC2* expression and that codon 90 is dispensable for translational arrest. Codon 89 was not fully essential for the induction of *yidC2*, though it might participate in stabilizing the translational arrest, because substitution of codon 89 by a stop codon showed intermediate *yidC2* expression. These data indicate that the translational arrest occurs near the MifM C-terminus, most likely after codon 88 is translated, consistent with the estimated molecular weight of the translational arrest product of GFP-MifM^{35–95} (~35 kDa, Figure 2B, band b). Translating ribosomes cover approximately 6–9 nucleotides of mRNA to the 3' of the A site (Culver, 2001; Yusupova *et al*, 2001, 2006; Takyar *et al*, 2005), which would allow a ribosome arrested at codon 88 to partially cover the 5' side of the stem loop (see Figure 1C), thereby exposing the *yidC2* SD for the duration of the arrest.

Arrest requires amino acids within the ribosomal polypeptide exit tunnel

To identify amino acids required to arrest MifM translation, we mutagenized the C-terminal region of MifM. Codons 59–89 were targeted because the above results suggested that arrest occurred near codon 89, which would place this codon near the peptidyl transfer site of the ribosome, and because the C-terminal 30–40 amino acids of nascent polypeptides lie within the polypeptide exit tunnel of the ribosome and are therefore well positioned to mediate a translational arrest (Krieg *et al*, 1989; Mothes *et al*, 1994; Frank *et al*, 1995; Matlack and Walter, 1995; Ban *et al*, 2000; Daniel *et al*, 2008). We constructed a series of alanine replacements in GFP-MifM^{35–95}-FLAG and monitored accumulation of the arrest species by immunoblotting with GFP- and FLAG-specific antibodies. Changing I65, H68, R69, I70, W73, I74, M80, N81 to alanine clearly increased accumulation of full-length MifM (Figure 3E), suggesting they might be required for the arrest, whereas other substitutions had more subtle effects. These substitutions could affect protein or mRNA stability as well as translation, so we introduced identical substitutions in wild-type *mifM* in the context of *yidC2-lacZ* to assess the ability of these proteins to induce YidC2 in the *spoIIJ* strain. Substitutions at amino acids Y67, R69, I70, T71, W73, I74, M80 or N81 reduced YidC2 induction in the *spoIIJ* strain as expected for mutations that reduce the translational arrest (Figure 3F). Amino acids that both increased production of full-length MifM Δ TM and reduced *yidC2* induction in the *spoIIJ* strain allowed us to identify a minimal set of amino acids that are clearly critical for the arrest (R69, I70, W73, I74, M80, N81); these are upstream of the translational arrest that occurs at E88 or D89. Interestingly, substitutions at the site of the arrest (E88A, D89A) had only minor effects in either assay, indicating that the specific amino acid or

peptidyl-tRNA at the site at which translation arrests is not critical (although it is critical that translation continues beyond these amino acids, Figure 3D). The amino acids necessary for translational arrest lie within the polypeptide exit tunnel of the ribosome and distal to the peptidyl transferase centre (PTC) at which elongation occurs.

A mutation that alters the polypeptide exit tunnel of the ribosome compromises elongation arrest

The above results suggest that an interaction between the MifM nascent polypeptide and the ribosomal polypeptide exit tunnel arrests translation. If so, then mutations that alter the polypeptide exit tunnel could impair the interaction and prevent the translational elongation arrest. To isolate arrest-defective ribosomes, we took advantage of the similarity in the mode of action of MifM and erythromycin, which binds within the exit tunnel to inhibit polypeptide elongation (Schlunzen *et al*, 2003; Tu *et al*, 2005; Vazquez-Laslop *et al*, 2008). This effect can be prevented by mutations that change the amino acids and rRNA that line the exit tunnel, which convey erythromycin resistance (Erm^R; (Gaynor and Mankin, 2003; Zaman *et al*, 2007; Diner and Hayes, 2009). Certain Erm^R mutants in *E. coli* are defective in translational arrests mediated by regulatory nascent chains, such as the SecAYEG monitor SecM (Nakatogawa and Ito, 2002; Lawrence *et al*, 2008), the tryptophan sensor TnaC (Cruz-Vera *et al*, 2005), the erythromycin sensor ErmCL (Vazquez-Laslop *et al*, 2008) and the chloramphenicol sensor Crb^{cmIA} (Lawrence *et al*, 2008). We therefore isolated spontaneous Erm^R mutants from independent *B. subtilis* cultures. These mutants all had an identical duplication of seven amino acids in the L22 protein (⁹⁴SQINKRT¹⁰⁰; Figure 4A). On the basis of crystal structure of the ribosome (Ban *et al*, 2000), it is likely that this duplication causes a seven amino acid insertion within the interior of the ribosome exit tunnel (Figure 4A and E), in a region that can be mutated to Erm^R in other bacteria (Franceschi *et al*, 2004; Zaman *et al*, 2007).

We tested whether the Erm^R mutation affected YidC2 induction by introducing it into strains in which MifM Δ TM is encoded upstream of *yidC2-lacZ* (*mifM*(Δ TM)-*yidC2*^{6-lacZ}), which would normally express high levels of YidC2 because of the prolonged translational arrest caused by the absence of membrane insertion (Figure 1Ad). Expression of *yidC2-lacZ* was drastically decreased in the Erm^R strain compared with wild type (Figure 4B). However, when the stem-loop mutation was introduced to relieve the dependence of *yidC2* expression on translational arrest (*mifM*-*yidC2*^{6-lacZ}(Δ stem): Figure 1Df), the Erm^R strain showed wild-type expression. Thus, the Erm^R mutation does not affect translation or folding of β -galactosidase. We next used GFP-MifM^{35–95}-FLAG to directly compare levels of the arrest and full-length species by immunoblotting. In the Erm^R mutant full-length MifM was the major species with only a small amount of the arrest product accumulated (Figure 4C, lanes 3 and 6), the opposite of the wild-type strain in which little full-length MifM accumulated (lanes 2 and 5). Thus, a mutation in the polypeptide exit tunnel of the ribosome compromises both MifM translational arrest and *yidC2* induction. We conclude that the elongation arrest of MifM is mediated by interaction between the C-terminal region of MifM and the polypeptide exit tunnel of the ribosome (Figure 4E).

Figure 4 A mutation affecting the ribosomal protein L22 compromises elongation arrest (**A**) The spontaneous *Erm*^R mutant has a seven amino acid duplication in L22 (white letters on black). Alignment of this region of *B. subtilis* (*Bs*) and *E. coli* (*Ec*) L22; underlined amino acids affect elongation arrest of *E. coli tnaC* (K90; Cruz-Vera *et al*, 2005) or *secM* (G91 and A93; Nakatogawa and Ito, 2002). (**B**) β -gal activity of *mifM*(Δ TM)-*yidC2*⁶-*lacZ* (left) or the stem-loop mutant (right) in *Erm*^R (black) and wild-type (white) strains. (**C**) Immunoblotting of GFP–MifM^{35–95}–FLAG in the *Erm*^R mutant with GFP and FLAG-specific antibodies. Strains with (2, 3, 5, 6) or without GFP fusion (1, 4) in wild type (lanes 1, 2, 4 and 5) or *Erm*^R (3, 6). *, non-specific bands (**D**) Synthetic cold sensitive growth is observed in the *erm spoIIIJ* double mutant. Strains were streaked on LB plates and incubated at the indicated temperature (RT, room temperature $\sim$ 24°C). (**E**) View of the ribosome polypeptide exit tunnel, showing approximate positions of L22 (black arrowheads) and MifM (white arrowheads) amino acids required for arrest. Dotted circle shows PTC.

During the course of these studies we noted that the arrest-defective *Erm*^R strain showed a cold sensitive growth defect in the *spoIIIJ* background, whereas the single *erm* or *yidC2* *erm* double mutant showed only a minor growth phenotype (Figure 4D). We surmise that the *Erm*^R mutant expresses sufficient YidC2 to support growth in the absence of SpoIIIJ at high temperatures, likely because of the residual translational arrest observed in western blots (Figure 4C). However, the reduced arrest activity prevents YidC2 from being fully induced, thereby limiting YidC2 activity and resulting in cold sensitive growth, as is also observed for *E. coli* mutants in the *sec* genes (Pogliano and Beckwith, 1993) and *yidC* (Yuan *et al*, 2007). These results suggest that erythromycin resistance might decrease the ability of a bacterium to survive events that compromise membrane protein insertion.

Phylogenetic distribution of *yidC2* and *mifM*

Our results indicate that MifM allows *B. subtilis* cells to produce additional YidC2 protein when the activity or level

of the primary YidC protein (SpoIIIJ) is reduced. We performed a phylogenetic analysis to determine how many bacteria have two copies of *yidC* and which of these were in an operon with a *mifM*-like gene. Our analysis indicated that the genomes of most Gram-negative bacteria had just one copy of *yidC*, whereas the genomes of many Gram-positive bacteria had more than one (Figure 5A; Supplementary Table S1). However, not all Gram-positive bacteria encode two YidC proteins. Within the phylum Firmicutes, most Lactobacillales have two *yidC* genes (for an average of 1.98 *yidC* genes/genome), whereas most Clostridiales have one (for an average of 1.14 *yidC* genes/genome). Most species within the order Bacillales have two *yidC* genes (for an average of 1.8 *yidC* genes/genome). *Staphylococcus aureus* is a notable exception in *Staphylococci* sp. as it encodes just one *yidC* gene. We were unable to detect MifM in the Actinobacteria, the Lactobacillales or the Clostridiales. Within the Bacillales, *mifM*-like genes were identified upstream of *yidC2* in

Figure 5 Phylogenetic distribution of *yidC2* and *mifM* in selected bacterial groups. **(A)** Average number of *yidC* genes in selected bacterial genera. Those shown in grey have five or fewer sequenced species. **(B)** Plot showing the distribution of *yidC2* in different bacterial genomes. *Clostridium kluyveri* has three *yidC* genes. **(C)** Alignment of the MifM with SecM and TnaC aligning the proteins from the C-terminal amino acid in the arrest product. Blue (acidic amino acids), Red (basic amino acids), stars, sites at which mutations reduce the arrest activity.

Bacillus sp., *Listeria* sp. and *Geobacillus* sp. (Figure 5B; Supplementary Table S2) but absent from the genomes of *Staphylococci* sp. that encoded both *yidC1* and *yidC2*. The restriction of the *mifM*–*yidC2* system to a subset of the Bacillales is similar to the distribution of the *secM*–*secA* system, which is restricted to a subset of the γ -proteobacteria (van der Sluis and Driessen, 2006).

Discussion

Our results support the following model for the mechanism by which *B. subtilis* upregulates its secondary membrane protein insertase (YidC2) when activity of the primary membrane protein insertase (SpoIIJ/YidC1) is limited. When membrane protein secretion capacity is high, *yidC2* translation is repressed by a stem loop that masks the *yidC2* SD, thereby inhibiting *yidC2* translation (Figure 6A and I). However, when membrane insertion of MifM is prevented by a *spoIIJ* mutation or by deletion of the MifM TM segment, *mifM* translation is arrested. The arrested ribosome is positioned over the 5' half of the stem loop, thereby exposing the *yidC2* SD and allowing *yidC2* translation (Figure 6A and V). This regulatory mechanism requires two distinct communication events between the MifM nascent polypeptide, the ribosome and the membrane insertion apparatus. First, our genetic data indicate that translational arrest requires an interaction between the arrest motif near the MifM C-terminus and the polypeptide exit tunnel of the ribosome. This interaction within the ribosome must be communicated back to the peptidyl transferase site at which elongation occurs. Second, the translational arrest is relieved by an event that is likely to be the insertion of MifM into the membrane, as it requires the N-terminal TM domain of MifM and SpoIIJ (YidC1). This event must also be communicated back to the peptidyl transferase site to allow continued elongation. The MifM–YidC2 system provides the first example of a mechanism in which a bacterial cell senses the activity of the YidC

pathway and the second example of a regulatory nascent chain that senses interactions outside the ribosome.

Many regulatory nascent chains are short polypeptides encoded in the 5' end of an operon. These short nascent chains mediate translational arrest in response to a cofactor (amino acid or antibiotic) that directly or indirectly modifies the interaction between the nascent chain and the ribosome to mediate stalling (reviewed by Yanofsky, 1981; Yanofsky *et al*, 1996; Tenson and Ehrenberg, 2002). This translational arrest can govern either transcription or translation of downstream genes. For example, the *E. coli* *tna* operon, which controls tryptophan catabolism, is regulated by a translational arrest within the 5' *tnaC* gene, which encodes a polypeptide of 24 amino acids. Translation of *tnaC* is arrested when tryptophan concentration increases and free tryptophan binds to the ribosome, stalling translation (Tenson and Ehrenberg, 2002; Cruz-Vera *et al*, 2005; Yanofsky, 2007). The stalled ribosome prevents Rho-dependent transcriptional termination, thereby inducing the downstream genes. Operons conferring resistance to antibiotics that target the ribosome often are regulated in a manner that directly parallels the mechanism by which *yidC2* is regulated. For example, the *ermC* gene (which confers erythromycin resistance) is regulated by a short upstream ORF in which translation is arrested in the presence of erythromycin (Narayanan and Dubnau, 1987). This arrest unfolds a stem loop thereby revealing the *ermC* SD sequence and allowing translation of *ermC*, which confers erythromycin resistance. Common features of the short regulatory nascent chains described to date are that they respond to binding of antibiotics or cofactors within the ribosome and that they are short polypeptides that are mostly accommodated within the ribosome.

MifM provides the second example of a long regulatory nascent chain that monitors an event sensed outside the ribosome. The first to be described is *E. coli* SecM, which monitors protein secretion through the SecYEG pathway and upregulates the SecA protein translocation ATPase when

Figure 6 Model for the *B. subtilis* membrane protein insertion monitor MifM and comparison to the *E. coli* protein secretion monitor SecM. (A) Model for YidC2 regulation. The mRNA hairpin masks the *yidC2* SD site to block *yidC2* translation (I). When MifM inserts into the membrane, the translational arrest is transient or does not occur, thereby allowing only transient unfolding of the mRNA secondary structure by the ribosome (II–IV). When membrane insertion of MifM is impaired, *mifM* translation is arrested and the ribosome unfolds the mRNA, allowing ribosomes to recognize the *yidC2* SD and initiate translation (V). (B) Comparison to the protein secretion monitor *secM*. Formation of the mRNA hairpin masks the *secA* SD and blocks *secA* translation. When SecM is secreted, the translational arrest is released, transiently exposing the *secA* SD and basal levels of SecA are synthesized (I–IV). When secretion is impaired, SecM is not secreted and a prolonged translational arrest occurs (V), unfolding the mRNA and allowing elevated SecA synthesis. (C) Proposed modular arrangement of MifM and SecM. The N-terminus of each protein contains a targeting signal comprised of either the putative transmembrane domain of MifM, which likely directs the protein to the SpoIIJ (YidC1) membrane protein biogenesis pathway, or the signal sequence of SecM, which directs the protein to the SecYEG protein secretion pathway. Positive and negative charges flanking the hydrophobic core of these signals are indicated. The C-terminus of each protein contains a translational arrest motif comprising ~12–20 amino acids preceding the final amino acid required for translational arrest (indicated by a D or P). This motif is located within the polypeptide exit tunnel of the ribosome, whereas the final amino acid translated is a peptidyl–tRNA at the PTC. TnaC is shown for comparison.

protein secretion capacity is limiting (Oliver and Beckwith, 1982; Schmidt and Oliver, 1989). The mechanism by which SecM monitors protein export is strikingly similar to the mechanism by which MifM monitors membrane protein insertion. Specifically, under conditions of limiting protein secretion capacity or when SecM secretion is blocked by deletion of the signal peptide, *secM* translation is arrested (McNicholas *et al*, 1997; Nakatogawa and Ito, 2001, 2002). This arrest positions the ribosome over the 5' side of a stem loop that occludes the SecA SD sequence, allowing increased *secA* translation (Figure 6B). Similar to *mifM*, *secM* encodes a protein with a C-terminal motif that interacts with the exit tunnel of the ribosome to induce a translational arrest (Figure 6C). Both proteins also include an N-terminal targeting motif that dictates the cellular pathway that the regulatory nascent chain monitors: SecM has an N-terminal signal peptide that directs the protein to the SecYEGA translocase for movement across the cytoplasmic membrane, whereas MifM has a single predicted TM segment and an intracellular

domain. These two proteins are likely moved across the membrane by separate cellular pathways, as SecM is secreted by the SecYEGA preprotein translocase, whereas the predicted topology of MifM is similar to that of *E. coli* proteins that are inserted into the bilayer in a YidC-dependent but SecYEGA-independent manner (Xie and Dalbey, 2008). These results suggest that long regulatory nascent chains have a modular arrangement, with the N-terminal domain specifying which event outside the ribosome is monitored and a C-terminal arrest motif (Figure 6C). This modular structure raises the possibility that additional regulatory nascent chains that sense events outside the ribosome exist, such as chaperone-mediated protein folding or the assembly of protein complexes.

Our data indicate that the *mifM* translational arrest is mediated by specific interactions between the C-terminus of the MifM nascent chain and the polypeptide exit tunnel of the ribosome (Figure 4E), as is the case for other nascent chain-mediated translational arrest mechanisms. Similar to *tnaC*

and *secM*, *mifM* translational arrest depends on regions of the nascent chain that are located away from the PTC at which translation is arrested, within in the polypeptide exit tunnel (Gong and Yanofsky, 2002; Nakatogawa and Ito, 2002; Cruz-Vera *et al*, 2005, 2007). Furthermore, mutations that affect the L22 ribosomal protein, which forms a constriction in the middle of the exit tunnel, compromise *mifM* translational arrest as is the case for *secM*, *ermC* and *tnaC* (Nakatogawa and Ito, 2002; Cruz-Vera *et al*, 2005; Vazquez-Laslop *et al*, 2008). These data suggest that this region of the polypeptide exit tunnel senses arrest motifs and triggers a signal transduction event that inhibits polypeptide elongation at the PTC.

We were surprised to find no primary sequence similarity in the arrest motifs of MifM, SecM and TnaC (Figure 5C) and to find that genetic analysis revealed several differences between the architecture of the MifM arrest motif and these two well-studied examples. First, single amino acid substitutions near the site of the MifM translational arrest had little effect, whereas changing the C-terminal proline codons of *tnaC* and *secM* strongly inhibits translational arrest (Nakatogawa and Ito, 2002; Cruz-Vera and Yanofsky, 2008). Alignment of MifM homologs revealed a conserved cluster of negatively charged amino acids near the site of the translational arrest (Supplementary Table S2), so it is possible that arrest can occur at any of these sites. Second, no single amino acid substitution in MifM completely blocked the arrest, whereas a single substitution within SecM (at Arg¹⁶³, two amino acids away from the translational arrest) abolished translational arrest (Yap and Bernstein, 2009). Together these results suggest that MifM translational arrest might be mediated by multiple interactions that independently contribute to the arrest rather than by a limited number of critical amino acids. These differences in arrest motif architecture and the lack of sequence similarity suggest that there could be more than one mechanism to induce translational arrest and that many polypeptides might be capable of serving as regulatory nascent chains.

The release of the MifM and SecM translational arrests requires the interaction of the stalled ribosome with SpoIIJ (YidC1) or with the SecYEG protein translocation channel, respectively. This suggests that at least one substrate of the *B. subtilis* YidC homologues is targeted co-translationally, although it remains unclear if this is also the case for other substrates in *B. subtilis* or in *E. coli*. It is interesting that two different protein translocation machineries can mediate the release of similar translational arrests. It has been proposed that the translational arrest of SecM is released by a pulling force generated by the SecA ATPase motor during the translocation of the C-terminal domain of SecM across the membrane (Nakatogawa and Ito, 2002; Butkus *et al*, 2003; Nakatogawa *et al*, 2004). In this context, it is somewhat surprising that the MifM translational arrest is released by YidC-dependent membrane insertion, because the MifM C-terminus is likely to remain in the cytoplasm, so membrane insertion is not necessarily expected to generate a large pulling force. Furthermore, YidC-dependent membrane insertion can occur in the absence of an ATPase such as SecA (Samuelson *et al*, 2000; Serek *et al*, 2004), and only in some examples does YidC-mediated membrane protein insertion depend on the proton motif force or the signal recognition particle, which has GTPase activity (Facey *et al*, 2007; Celebi *et al*, 2008). Thus, although insertion of the TM segment into

the hydrophobic membrane might generate a local pulling force, it is unclear whether this force would be of similar magnitude as that generated by the ATP-driven export of a protein across the membrane. It is also possible that the release might be mediated by the direct interaction between the ribosome–nascent chain complex and membrane protein insertion apparatus, which could trigger signal transduction that rearranges the environment of the exit tunnel or the PTC so that the translational arrest of *mifM* is canceled. In keeping with this model, it has recently been shown that the ribosome–nascent chain makes nearly identical contacts with the YidC and SecYEG channels and that this interaction causes similar structural changes in the large subunit of the ribosome (Kohler *et al*, 2009). Additional studies are necessary to elucidate the precise mechanism for release of the MifM translational arrest.

Mitochondria, chloroplasts and certain Gram-positive bacteria express more than one YidC/Oxa1/Alb3 protein. In some cases, the YidC proteins are functionally differentiated. For example, mitochondrial Oxa1 has a C-terminal extension that interacts with the mitochondrial ribosome and thereby mediates cotranslational membrane insertion, whereas the homolog Cox18 lacks the C-terminal extension and functions at later steps of membrane protein insertion (reviewed by Bonnefoy *et al*, 2009). A similar functional differentiation has been proposed in the Gram-positive bacterium *S. mutans*, in which YidC2 possesses a C-terminal ribosome-docking site and is involved in co-translational membrane protein insertion, whereas YidC1 lacks this domain (Funes *et al*, 2009). Interestingly, neither *B. subtilis* SpoIIJ nor YidC2 has this C-terminal extension, although our data suggest that at least one SpoIIJ substrate, MifM, is targeted to SpoIIJ co-translationally. SpoIIJ and YidC2 clearly share an essential function, as it is possible to genetically inactivate one but not both genes. YidC2 therefore provides a backup for this essential function, allowing cells to survive an otherwise lethal event. The *mifM* sensing system we here describe allows this backup function to be inducible, perhaps reducing the fitness cost associated with expressing YidC2 when it is not needed. SpoIIJ and YidC2 also seem to have at least one non-overlapping function. Specifically, YidC2 is unable to substitute for SpoIIJ during sporulation (Errington *et al*, 1992; Murakami *et al*, 2002; Tjalsma *et al*, 2003), suggesting that certain membrane proteins might specifically require SpoIIJ for their biogenesis (such as SpoIIIAE; Camp and Losick, 2008). It is interesting to speculate that YidC2 might also be specifically required in a particular environment, or during a specific developmental pathway or stress response that is shared by those Gram-positive species that encode more than one YidC protein. We suspect that accessory YidC proteins might have different functions in different bacterial species, because not all *yidC2* genes are preceded by *mifM* (Figure 5B; Supplementary Table S2). This might also be the case for SecA and SecY, which are frequently duplicated in Gram-positive bacteria (Cao and Saier, 2003; Rigel and Braunstein, 2008; Siboo *et al*, 2008; Mistou *et al*, 2009).

Our phylogenetic analysis indicates that the membrane protein insertion monitor MifM evolved in a different group of bacteria than the protein secretion monitor SecM (van der Sluis and Driessen, 2006). This, together with the absence of sequence similarity in the arrest motif of the two proteins, suggests that MifM and SecM represent the parallel evolution

Table I Strains used in these studies

Strains	Genotype	Reference or source
PY79	wild type	Youngman <i>et al</i> (1984)
KP3034	<i>lacA::spec</i>	This study
KP11086	<i>amyE::yidC2²⁷⁵–lacZΩcat</i>	Rubio <i>et al</i> (2005)
KP11131	<i>amyE::yidC2²⁷⁵–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	Rubio <i>et al</i> (2005)
KP11268	<i>yidC2-gfpΩspc</i>	This study
SCB406	<i>amyE::mifM⁹⁵–lacZΩcat</i>	This study
SCB416	<i>amyE::mifM⁹⁵–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB421	<i>amyE::mifM⁵–lacZΩcat</i>	This study
SCB426	<i>amyE::mifM⁵–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB429	<i>yidC2-gfpΩspc, amyE::mifM(ΔTM)–yidC2⁶–lacZΩcat</i>	This study
SCB430	<i>yidC2-gfpΩspc, ΔspoIIIJ-jag::cat::tet</i>	This study
SCB454	<i>amyE::mifM(ΔTM)–yidC2⁶–lacZΩcat</i>	This study
SCB461	<i>amyE::mifM(ΔTM)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB467	<i>amyE::mifM(91-96rp)yidC2⁶–lacZΩcat</i>	This study
SCB474	<i>amyE::mifM(91-96rp)yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB495	<i>amyE::mifMyidC2⁶–lacZ(Δstem)Ωcat</i>	This study
SCB501	<i>amyE::mifMyidC2⁶–lacZ(Δstem)Ωcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB560	<i>amyE::mifM(D86stop)–yidC2⁶–lacZΩcat</i>	This study
SCB561	<i>amyE::mifM(A90stop)–yidC2⁶–lacZΩcat</i>	This study
SCB562	<i>amyE::mifM(frameshift)–yidC2⁶–lacZΩcat</i>	This study
SCB566	<i>amyE::mifM(D86stop)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB567	<i>amyE::mifM(A90stop)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB568	<i>amyE::mifM(frameshift)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB610	<i>ybaCΩloxP-kan-loxP</i>	This study
SCB625	<i>ybaCΩloxP-kan-loxP, erm^R</i>	This study
SCB663	<i>amyE::mifM(E87stop)–yidC2⁶–lacZΩcat</i>	This study
SCB664	<i>amyE::mifM(E88stop)–yidC2⁶–lacZΩcat</i>	This study
SCB665	<i>amyE::mifM(D89stop)–yidC2⁶–lacZΩcat</i>	This study
SCB669	<i>amyE::mifM(E87stop)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB670	<i>amyE::mifM(E88stop)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB671	<i>amyE::mifM(D89stop)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB675	<i>amyE::mifM(I66A)–yidC2⁶–lacZΩcat</i>	This study
SCB676	<i>amyE::mifM(Y67A)–yidC2⁶–lacZΩcat</i>	This study
SCB677	<i>amyE::mifM(H68A)–yidC2⁶–lacZΩcat</i>	This study
SCB678	<i>amyE::mifM(R69A)–yidC2⁶–lacZΩcat</i>	This study
SCB680	<i>amyE::mifM(T71A)–yidC2⁶–lacZΩcat</i>	This study
SCB681	<i>amyE::mifM(T72A)–yidC2⁶–lacZΩcat</i>	This study
SCB682	<i>amyE::mifM(W73A)–yidC2⁶–lacZΩcat</i>	This study
SCB683	<i>amyE::mifM(I74A)–yidC2⁶–lacZΩcat</i>	This study
SCB685	<i>amyE::mifM(K76A)–yidC2⁶–lacZΩcat</i>	This study
SCB686	<i>amyE::mifM(V77A)–yidC2⁶–lacZΩcat</i>	This study
SCB687	<i>amyE::mifM(F78A)–yidC2⁶–lacZΩcat</i>	This study
SCB688	<i>amyE::mifM(R79A)–yidC2⁶–lacZΩcat</i>	This study
SCB689	<i>amyE::mifM(I70A)–yidC2⁶–lacZΩcat</i>	This study
SCB689	<i>amyE::mifM(M80A)–yidC2⁶–lacZΩcat</i>	This study
SCB691	<i>amyE::mifM(S82A)–yidC2⁶–lacZΩcat</i>	This study
SCB692	<i>amyE::mifM(P83A)–yidC2⁶–lacZΩcat</i>	This study
SCB693	<i>amyE::mifM(V84A)–yidC2⁶–lacZΩcat</i>	This study
SCB694	<i>amyE::mifM(N85A)–yidC2⁶–lacZΩcat</i>	This study
SCB695	<i>amyE::mifM(D86A)–yidC2⁶–lacZΩcat</i>	This study
SCB696	<i>amyE::mifM(E87A)–yidC2⁶–lacZΩcat</i>	This study
SCB697	<i>amyE::mifM(E88A)–yidC2⁶–lacZΩcat</i>	This study
SCB705	<i>amyE::mifM(D86silent)-TAA-yidC2⁶–lacZΩcat</i>	This study
SCB706	<i>amyE::mifM(I66A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB707	<i>amyE::mifM(Y67A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB708	<i>amyE::mifM(H68A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB709	<i>amyE::mifM(R69A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB710	<i>amyE::mifM(I70A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB711	<i>amyE::mifM(T71A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB712	<i>amyE::mifM(T72A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB713	<i>amyE::mifM(W73A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB714	<i>amyE::mifM(I74A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB716	<i>amyE::mifM(K76A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB717	<i>amyE::mifM(V77A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB718	<i>amyE::mifM(F78A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB719	<i>amyE::mifM(R79A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB720	<i>amyE::mifM(M80A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB722	<i>amyE::mifM(S82A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB723	<i>amyE::mifM(P83A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB724	<i>amyE::mifM(V84A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB725	<i>amyE::mifM(N85A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB726	<i>amyE::mifM(D86A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB727	<i>amyE::mifM(E87A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB728	<i>amyE::mifM(E88A)–yidC2⁶–lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study

Table 1 Continued

Strains	Genotype	Reference or source
SCB736	<i>amyE::mifMD86(silent)-TAA-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB751	<i>amyE::yidC2⁶-lacZΩcat</i>	This study
SCB757	<i>amyE::yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB762	<i>amyE::mifM(N81A)-yidC2⁶-lacZΩcat</i>	This study
SCB767	<i>amyE::mifM(N81A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB803	<i>amyE::mifM(T60A)-yidC2⁶-lacZΩcat</i>	This study
SCB804	<i>amyE::mifM(D61A)-yidC2⁶-lacZΩcat</i>	This study
SCB805	<i>amyE::mifM(F62A)-yidC2⁶-lacZΩcat</i>	This study
SCB806	<i>amyE::mifM(L63A)-yidC2⁶-lacZΩcat</i>	This study
SCB807	<i>amyE::mifM(I65A)-yidC2⁶-lacZΩcat</i>	This study
SCB814	<i>amyE::mifM(T60A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB815	<i>amyE::mifM(D61A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB816	<i>amyE::mifM(F62A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB817	<i>amyE::mifM(L63A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB818	<i>amyE::mifM(I65A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB828	<i>amyE::rbsm1-gfp-mifM³⁵⁻⁹⁵-yidC2⁶-lacZΩcat</i>	This study
SCB832	<i>amyE::mifM(R75A)-yidC2⁶-lacZΩcat</i>	This study
SCB839	<i>amyE::mifM(R75A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB844	<i>amyE::yidC2⁶-lacZΩcat, lacA::spc</i>	This study
SCB846	<i>amyE::mifM(R59A)-yidC2⁶-lacZΩcat</i>	This study
SCB847	<i>amyE::mifM(D89A)-yidC2⁶-lacZΩcat</i>	This study
SCB851	<i>amyE::rbsm1-gfp-mifM³⁵⁻⁹⁵-flag-yidC2⁶-lacZΩcat</i>	This study
SCB858	<i>amyE::mifM(R59A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB859	<i>amyE::mifM(D89A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB966	<i>ybaCΩloxP-kan-loxP, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB967	<i>ybaCΩloxP-kan-loxP, erm^R, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB968	<i>ybaCΩloxP-kan-loxP, ΔyidC2::spc</i>	This study
SCB969	<i>ybaCΩloxP-kan-loxP, erm^R, ΔyidC2::spc</i>	This study
SCB972	<i>amyE::mifM(ΔTM)-yidC2⁶-lacZΩcat, ybaCΩloxP-kan-loxP</i>	This study
SCB973	<i>amyE::mifM(ΔTM)-yidC2⁶-lacZΩcat, ybaCΩloxP-kan-loxP, erm^R</i>	This study
SCB976	<i>amyE::P_{mifM}-rbsm1-gfp-mifM³⁵⁻⁹⁵-flag-yidC2⁶-lacZΩcat, ybaCΩloxP-kan-loxP</i>	This study
SCB993	<i>amyE::P_{mifM}-rbsm1-gfp-mifM³⁵⁻⁹⁵-flag-yidC2⁶-lacZΩcat, ybaCΩloxP-kan-loxP, erm^R</i>	This study
SCB994	<i>amyE::mifM(Δstem)yidC2⁶-lacZΩcat, ybaCΩloxP-kan-loxP</i>	This study
SCB995	<i>amyE::mifM(Δstem)yidC2⁶-lacZΩcat, ybaCΩloxP-kan-loxP, erm^R</i>	This study
SCB996	<i>amyE::mifM(I64A)-yidC2⁶-lacZΩcat</i>	This study
SCB1002	<i>amyE::mifM(I64A)-yidC2⁶-lacZΩcat, ΔspoIIIJ-jag::cat::spc</i>	This study
SCB1044	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵-flagΩcat</i>	This study
SCB1045	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(R75A)-flagΩcat</i>	This study
SCB1049	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(I74A)-flagΩcat</i>	This study
SCB1051	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(R59A)-flagΩcat</i>	This study
SCB1052	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(T60A)-flagΩcat</i>	This study
SCB1053	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(D61A)-flagΩcat</i>	This study
SCB1054	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(F62A)-flagΩcat</i>	This study
SCB1055	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(L63A)-flagΩcat</i>	This study
SCB1056	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(I64A)-flagΩcat</i>	This study
SCB1057	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(I65A)-flagΩcat</i>	This study
SCB1058	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(I66A)-flagΩcat</i>	This study
SCB1059	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(Y67A)-flagΩcat</i>	This study
SCB1060	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(H68A)-flagΩcat</i>	This study
SCB1061	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(R69A)-flagΩcat</i>	This study
SCB1062	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(I70A)-flagΩcat</i>	This study
SCB1064	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(T72A)-flagΩcat</i>	This study
SCB1065	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(W73A)-flagΩcat</i>	This study
SCB1066	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(K76A)-flagΩcat</i>	This study
SCB1067	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(V77A)-flagΩcat</i>	This study
SCB1068	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(F78A)-flagΩcat</i>	This study
SCB1069	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(R79A)-flagΩcat</i>	This study
SCB1070	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(M80A)-flagΩcat</i>	This study
SCB1071	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(N81A)-flagΩcat</i>	This study
SCB1072	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(S82A)-flagΩcat</i>	This study
SCB1073	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(P83A)-flagΩcat</i>	This study
SCB1074	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(V84A)-flagΩcat</i>	This study
SCB1075	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(N85A)-flagΩcat</i>	This study
SCB1076	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(D86A)-flagΩcat</i>	This study
SCB1077	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(E87A)-flagΩcat</i>	This study
SCB1078	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(E88A)-flagΩcat</i>	This study
SCB1260	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(T71A)-flagΩcat</i>	This study
SCB1261	<i>amyE::P_{mifM}-gfp-mifM³⁵⁻⁹⁵(D89A)-flagΩcat</i>	This study
ALB185	<i>spoIIIJ-flagΩloxP-kan-loxP, lacA::spec</i>	This study
ALB198	<i>spoIIIJ (Shine–Dalgarno AGGAGG to AGGAAG), amyE::yidC2⁶-lacZΩcat, lacA::spec</i>	This study
ALB228	<i>spoIIIJ (Shine–Dalgarno AGGAGG to AAGAGG), amyE::yidC2⁶-lacZΩcat, lacA::spec</i>	This study
ALB396	<i>spoIIIJ (Shine–Dalgarno AGGAGG to AGAAGG), amyE::yidC2⁶-lacZΩcat, lacA::spec</i>	This study

of protein sensors of two different pathways that act by similar molecular mechanisms. The increasing numbers of regulatory nascent chains that lack primary sequence identity suggests that the ribosome does not blindly translate the genetic code. Translation instead seems to represent an on-going negotiation between the ribosome, the nascent polypeptide and other cellular components such as the machineries for membrane protein biogenesis and protein secretion. Although this might limit the usage of the genetic code, it allows cells to use nascent chains as regulatory switches that respond to different co-translational or translational events. Clearly nascent chains are not simply non-functional intermediates in proteins synthesis and instead are capable of interacting with a discriminating ribosome to provide regulatory mechanisms that enable cells to respond to changing intracellular environments.

Materials and methods

Strain and plasmid construction

B. subtilis strains (Table I) were constructed by transformation (Hoch, 1991). The $\Delta spoIIIJ$ -*jag* deletion strain used in these studies is a complete deletion in which the coding region was replaced by a *cat* gene that was subsequently replaced with the *tet* gene (Rubio *et al.*, 2005) using pCm::Tc (Steinmetz and Richter, 1994). The *lacA::spec* mutation (Hartl *et al.*, 2001) was transformed into PY79 for these studies. Plasmids (Supplementary Table S3) were constructed by standard cloning methods, PCR and site-directed mutagenesis (Sawano and Miyawaki, 2000) using the strategies described in Supplementary data and the oligonucleotides in Supplementary Table S4. Plasmids were verified by sequencing the coding region. Following introduction into the *B. subtilis* chromosome, recombinants were checked for their antibiotic resistance, the inactivation of *amyE* and the loss of any additional drug resistance markers on the plasmid backbone.

Growth conditions and general procedures

B. subtilis cells were cultured at 37°C in LB and CH media (Sterlini and Mandelstam, 1969) for western blotting and β -galactosidase activities assay, respectively. Cultures with OD₆₀₀ = 0.5–1.0 were collected for each experiment. β -galactosidase assays (Miller, 1972; Rubio *et al.*, 2005) and immunoblots (Blaylock *et al.*, 2004) were performed as described earlier. Blots were probed with monoclonal anti-GFP (Roche), and either polyclonal (Figure 3F) or monoclonal (Figures 2 and 5) anti-FLAG (Sigma).

Isolation of mutants that overexpress *yidC2*

A 10 ml aliquot of SCB844 (*amyE::yidC2⁶-lacZ Ω cat*, *lacA::spec*) culture with OD₆₀₀ = 0.5 in LB media was collected, washed with the citrate buffer (100 mM citric acid, pH 5.5) twice, suspended in 10 ml of the citrate buffer and divided into 10 tubes. The cells were treated with 0.1 mg/ml NTG for 30 min at 30°C, then washed with 1 ml of phosphate buffer (pH 7.0) and suspended in 1 ml of LB followed by addition of the same volume of 50% glycerol. The mutant pools were stored at –80°C until subjected to screening. The mutant pool was diluted and aliquots spread on DSM plates containing 60 μ g/ml X-gal. Blue colonies were isolated at 37 and 42°C. Chromosomal DNA of Lac⁺ mutants was isolated and transformed into PY79 selecting for Cm^R encoded by *amyE::yidC2⁶-lacZ Ω cat* to identify mutations linked to the fusion. Unlinked mutations were moved into an unmutagenized background by

transformation into KP3034 (*lacA::spec*) competent cells using a high concentration of chromosomal DNA, selecting for Cm^R and isolating the rare blue colonies in which both the unknown mutation and the *lacZ* fusion had been co-transformed into the recipient. These backcrossed strains were checked for linkage to *spoIIIJ* by transformation with chromosomal DNA from strain ALB185 (Table I). The *spoIIIJ*-linked mutations were sequenced to identify the point mutations. Three different mutations affecting the SD sequence were identified (Supplementary data).

CTABr precipitation

CTABr precipitation was performed essentially as described (Gilmore *et al.*, 1991; Nakatogawa and Ito, 2001), with the following modifications. To prepare lysates of *B. subtilis* cells, 1 ml of LB culture was washed with 1 ml of HSM (20 mM HEPES (pH 7.6), 20% sucrose, 20 mM MgCl₂) buffer and treated with 1 mg/ml lysozyme in the same buffer for 10 min at 37°C. Cells were then collected by centrifugation and resuspended in 200 μ l of ice-cold 20 mM HEPES (pH 7.6), 150 mM NaCl and solubilized with 0.5% NP-40 on ice for 7 min. Insoluble particles were removed by centrifuge (4°C, 13 000 r.p.m. for 5 min) and 90 μ l of each cell lysate was mixed with 500 μ l of 2% CTABr and 500 μ l of 0.5 M NaOAc (pH 4.7) for CTABr precipitation. The mixture was incubated on ice for 10 min, then at 30°C for 10 min, centrifuged at 14 000 r.p.m. for 15 min and the pellet washed by acetone/HCl (19:1) twice and solubilized in SDS sample buffer. The supernatants were collected and proteins precipitated with 10% TCA and washed by acetone/HCl (19:1) before solubilization in SDS sample buffer. RNase treatment was done by adding 0.5 μ l of 4 mg/ml RNaseA into 90 μ l of cell lysate and incubated for 10 min at 37°C before addition of CTABr.

Isolation and characterization of erythromycin resistant (*Erm^R*) mutations

Strain SCB610 (*ybaC Ω loxP-kan-loxP*), which has a kanamycin resistance marker near the ribosomal protein operon was plated on LB plates with 2 μ g/ml of erythromycin to select spontaneous *Erm^R* mutants. Genetic linkage between the *erm* mutation and the ribosomal operon was tested by transforming chromosomal DNA of each mutant into PY79 (wild type) on LB erythromycin plates and screening for kanamycin resistance; all were linked. The *rplV* gene of wild type (SCB610) and *erm* mutants was PCR amplified and sequenced.

Microscopy and image analysis

For GFP visualization, live cells were stained with 5 μ g/ml FM 4–64 (Molecular Probes; (Sharp and Pogliano, 1999)). Images were collected using an Applied Precision optical sectioning microscope (Liu *et al.*, 2006) and deconvolved using softWoRx v3.3.6 (Applied Precision).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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