Error-prone initiation factor 2 mutations reduce the fitness cost of antibiotic resistance

Introduction

Initiation of protein synthesis takes place after splitting the post-termination 70S ribosome into its small (30S) and large (50S) subunits. The ribosome splitting, which is catalysed by ribosome recycling factor (RRF) and elongation factor G (EF-G), is followed by the binding of initiation factor 3 (IF3) to the 30S subunit (Karimi et al., 1999; Peske et al., 2005; Pavlov et al., 2008). Subsequent binding of initiation factors 1 (IF1) and 2 (IF2), messenger RNA (mRNA) and initiator tRNA (formyl(f)-Met-tRNA) to the 30S:IF3 complex results in the formation of the 30S pre-initiation complex (30S PIC) (Gualerzi et al., 2001; Antoun et al., 2006a).

Correct positioning of the start codon of mRNAs in the P site of the 30S subunit requires the presence of fMet-tRNA (rather than an elongator tRNA) in the 30S PIC (Hartz et al., 1989). The accuracy of initiator tRNA selection into the 30S PIC is greatly enhanced by IF1, IF2 and IF3 (Wintemeyer and Gualerzi, 1983; Pon and Gualerzi, 1984; Canonaco et al., 1986; Antoun et al., 2006a,b). IF3 blocks premature docking of the 50S subunit to an initiator tRNA-less 30S PIC and increases the rate constants for tRNA association to, and dissociation from, the 30S subunit (Subramanian and Davis, 1970; Gualerzi et al., 2001; Lancaster and Noller, 2005; Antoun et al., 2006a).

IF2 plays a pivotal role in the fast binding of fMet-tRNA into the 30S PIC (Benne et al., 1973; Fakundy and Hershey, 1973; Wintemeyer and Gualerzi, 1983; Gualerzi et al., 2001; Antoun et al., 2006a) and the subsequent rapid docking of the 50S subunit to the 30S PIC containing fMet-tRNA (Antoun et al., 2003, 2006a; Grigoriadou et al., 2007), thereby ensuring high accuracy of initiator tRNA selection into the 70S initiation complex (Antoun et al., 2006b). IF1 together with IF2 and IF3 enhances the accuracy of initiator tRNA selection by selectively increasing the rate of fMet-tRNA binding to the 30S subunit (Pon and Gualerzi, 1984; Antoun et al., 2006b).

The formyl group of fMet-tRNA greatly increases the ability of IF2 to distinguish between initiator tRNA and aminoacylated elongator tRNAs (Sundari et al., 1976; Gualerzi et al., 2001; Antoun et al., 2006b). Formylation of Met-tRNA is catalysed by the formyl-methionine-
transferase (FMT), which recognizes a C1·A72 mismatch present in tRNA, but absent in most bacterial elongator tRNAs (RajBhandary, 1994). Formylation of the methionine of initiator tRNA and subsequent removal of the formyl group from finished proteins by peptide deformylase (PDF) occur in most eubacteria as well as in mitochondria and chloroplasts of eukaryotes (Solbiati et al., 1999; Vaughan et al., 2002). Formylation of formylated proteins by PDF is often required for their activity (Solbiati et al., 1999; Bingel-erlenmeyer et al., 2008), making this enzyme an attractive target for novel antimicrobial drugs (Vaughan et al., 2002). One example is PDF inhibitors such as actinonin that cause accumulation of non-functional formylated proteins in the cell, ultimately leading to arrested cell growth (Chen et al., 2000).

Mutations abolishing formylation of Met-tRNAi confer resistance to actinonin since bypass of the formylation step makes the de-formylation step and hence the PDF activity redundant (Apfel et al., 2001). Formylation deficiency in Enterobacteriaceae leads to very slow growth, showing that formylation is required for fast growth but not for cell viability (Guillon et al., 1992; 1996; Steiner-Mosonyi et al., 2004). However, resistant bacteria can reduce the fitness cost associated with formylation deficiency by acquiring compensatory mutations at maintained resistance (Andersson and Levin, 1999; Andersson, 2006). Second-site mutations that increase the growth rate of formylation-deficient bacteria have previously been found (Margolis et al., 2000; Nilsson et al., 2006). For example, in S. typhimurium lack of a functional FMT enzyme can be efficiently compensated by increasing the Met-tRNAi concentration via high-level copy-number amplification of the tRNA genes metZ and metW (Nilsson et al., 2006).

Here we identified novel mutations in IF2 that partially compensated for the formylation deficiency in actinonin resistant strains with a normal concentration of initiator tRNA. IF2 consists of four structural domains (Roll-Mecak et al., 2000), with the initiator tRNA-binding domain IV connected to domain III by a long helical linker (Roll-Mecak et al., 2000; Allen et al., 2005; Simonetti et al., 2008). Previously, it was demonstrated that the activity of IF2 in initiation with non-formylated Met-tRNA, can be increased by specifically engineered mutations in domain IV that increase the affinity of IF2 for Met-tRNA (Steiner-Mosonyi et al., 2004). Unexpectedly, none of the IF2 compensatory mutations identified here were located in domain IV, indicating that they do not compensate the formylation deficiency by increasing the IF2 affinity for Met-tRNA. Instead, this new class of IF2 mutants appear to increase the rate of initiation with Met-tRNA by increasing the propensity of IF2 to adopt the 50S docking conformation on the 30S ribosomal subunit not only in the presence of fMet-tRNA, but also with Met-tRNA, deacylated tRNA, and elongator tRNAs.

We propose that the IF2 mutants with the strongest compensatory effect have reduced growth rates in a formylation-proficient background due to a highly increased frequency of aberrant initiation events. Importantly, from the observed linear correlation between our biochemical data on the initiation time and the measured bacterial generation time, we suggest that the rate of bulk protein synthesis in the cell is mRNA limited, leading to hypersensitive variation in growth rate in response to variation in initiation rate.

**Results**

**In vivo analysis**

We previously subjected five different formylation-deficient and slow-growing actinonin-resistant fmt mutants to compensatory evolution to select for mutants with increased growth rate (Nilsson et al., 2006). After 50–150 generations of growth, fast-growing mutants were recovered and their compensatory mutations were identified as either point mutations in fmt, amplification of the tRNA genes metZ and metW or as an unknown class of mutations (Nilsson et al., 2006). In the present study we have identified the compensatory mutations in the latter class as point mutations in the infB gene, coding for initiation factor 2 (IF2). Transfer of the five unique point mutations into a formylation-deficient (fmt mutant) strain confirmed their growth compensatory nature (Table 1; Fig. 1).

We then extended the search for growth compensating IF2 mutants by performing localized hydroxylamine mutagenesis of infB. Phage P22 grown on a strain with a transposon inserted near infB was isolated, mutagenized with hydroxylamine and used to transduce a slow-growing fmt mutant strain. By screening for fast growers among the tetracycline resistant transductants, nine individual compensated mutants were isolated and their infB genes were sequenced. Eight novel infB mutations were found (Table 1) and as eight out of the nine mutations were recovered only once, this indicates that the mutational target is not saturated with compensatory mutations. In total, 13 different IF2 mutations, all located well outside tRNA binding domain IV of IF2, have been identified (Table 1; Fig. 2). Cryo-EM studies show also that none of the IF2 mutations could have any direct contact with fMet-tRNA in the 30S pre-initiation and the 70S initiation complexes (Allen et al., 2005; Myasnikov et al., 2005; Simonetti et al., 2008). It is therefore highly unlikely that any of the mutations in IF2 isolated here conferred growth compensation simply by increasing the affinity of IF2 to Met-tRNA.
Growth rates of IF2 mutants in a formylation-deficient background

We used linkage to a nearby transposon to transfer six different IF2 mutations into the same fmt mutant background to compare the growth rate in rich medium for each one of the different infB mutations with the wild-type in an isogenic strain background. The tested mutants could be roughly divided into two classes, one with high (A1–A3) and one with low (B1–B3) compensatory effect. Figure 2 and Table 1 show that the class A compensatory mutations are located in neighbouring helices H10 (A3) and H11 (A1 and A2) of the domain III, while the class B mutations are located in domain III (B1), in the helical linker between domains III and IV (B2) and in the G-domain (B3) of IF2. The bacteria in both classes grew faster than the original fmt mutant with wild-type IF2 (Fig. 1).

To further verify that the infB mutations and resulting amino acid substitutions in IF2 were solely responsible for the observed fitness-increasing phenotype, a complementation test was performed. The infB mutant genes were cloned into the vector pBAD30, transformed into the fmt mutant strain and growth rates were measured. As expected, growth rates were improved but were slightly lower than the previously measured mutant growth rates (Fig. 1). This small reduction in the compensatory effect of the mutations could be due to the presence of wild-type IF2 in these complemented strains.

Growth rates of IF2 mutants in a formylation-proficient background

We compared the growth rate of a strain with wild-type IF2 to the growth rates of the IF2 mutants in an otherwise wild-type background in rich media and in minimal media supplemented with either glucose or glycerol. The class A IF2 mutants grew 10–25% slower than the wild-type strain, whereas the class B mutants showed a smaller (<5%) growth rate reduction (Fig. 3). For both mutant classes, the fitness reduction was higher in poor than in rich media (Fig. 3). To confirm that the fitness effects seen in vivo resulted from altered activity of the mutant IF2 rather than from alterations in the intracellular level of mutant protein, we measured IF2 levels during exponential growth using Western blotting. No difference in the

| Strain number | Mutation | Location in IF2 structure | IF2 mutant |
|---------------|----------|---------------------------|------------|
| DA10610       | A740V, gcg→gtg | Domain III, H10          | B1         |
| DA8781        | S741F, tgt→ctc   | Domain III, H10          |           |
| DA8836        | R751L, cgt→ctt   | Domain III               |           |
| DA10710       | A783V, gcg→gtg | Linker, H12              | B2         |
| DA10609       | S755Y, tct→tat   | Domain III, H11          | A1         |

Hydroxylamine mutagenesis

| Strain number | Mutation | Location in IF2 structure | IF2 mutant |
|---------------|----------|---------------------------|------------|
| DA13317       | A740V, gcg→gtg | Domain III, H10 (B1)*   | A1         |
| DA13318       | A783T, gcc→acg | Linker, H12              |           |
| DA13363       | A182T, gct→act | N-Domain                 |           |
| DA13368       | A393V, gcc→gtc | G-domain                 | B3         |
| DA13369       | E763K, gaa→aaa | Domain III               |           |
| DA14394       | E732K, gaa→aaa | Domain III, H10          | A3         |
| DA14395       | A752T, gcc→acc | Domain III               |           |
| DA14396       | S755F, tct→tt  | Domain III, H11          | A2         |
| DA14397       | A484V, gct→gtt | G-domain, H4             |           |

* This mutation was found twice.

Both the nucleotide change and the resulting amino acid change are shown. Mutant strains further tested in vitro are marked in bold. Location of the mutations in IF2 structure follows the nomenclature used in Roll-Mecak et al. (2000).

Fig. 1. Comparison of growth rates for the formylation-deficient strains with compensatory IF2 mutations, original Fmt- strain and formylation-proficient Fmt+ wild-type strain. Black bars represent strains harbouring the IF2 mutation on the chromosome, light grey bars represent the IF2-mutants on plasmid pBAD30. Mutants are grouped as class A (strongly compensating) and class B (weakly compensating).
steady-state level of IF2 could be seen between the wild-type, the parental \textit{fmt} mutant and the various IF2 mutants (data not shown).

\textbf{In vitro analysis}

Two steps in initiation of translation depend crucially on IF2: (i) binding of fMettRNA\textsubscript{i} to the mRNA-containing 30S subunit and (ii) subsequent docking of the 50S subunit to the complete 30S PIC (Hartz \textit{et al.}, 1991; Gualerzi \textit{et al.}, 2001; Antoun \textit{et al.}, 2006a,b). These two steps of initiation were mimicked in our biochemical experiments by rapidly mixing 50S subunits and initiator tRNAs with tRNA-lacking 30S PICs in a stopped flow instrument and monitoring the formation of 70S initiation complexes by Rayleigh light scattering (Antoun \textit{et al.}, 2003). The experiments were performed with wild-type IF2 and different IF2 mutants in combination with formylated, non-formylated or deacylated initiator tRNA as well as an aminoacylated elongator tRNA.

\textit{Initiation with IF2 mutants and formylated initiator tRNA}

Formation of 70S initiation complex after rapid mixing of 50S subunits and formylated initiator Met-tRNA\textsubscript{i} (fMettRNA\textsubscript{i}) with 30S PICs lacking tRNA proceeded with similar rates for wild-type and IF2 mutants (Fig. 4A). In the absence of initiator tRNA the rate and extent of 70S complex formation were very small, although significantly larger for class B mutants than for wild-type and significantly larger for class A than for class B mutants of IF2 (Fig. 4A). For simple quantification of the initiation rate ($k$), which involves initiator tRNA binding to active 30S PICs, subsequent docking of 50S subunits to tRNA-containing active 30S PICs and a slow conversion of a small fraction of the 30S PICs inactive in 50S docking into

\begin{figure}
\centering
\includegraphics[width=\textwidth]{structure.png}
\caption{The different point mutations isolated in this study highlighted in the structure of IF2. Class A mutations are marked in blue, other mutations are marked in red and \textit{in vitro} tested IF2 mutations are underlined. The A182T mutation is not shown because the structure does not contain the N-terminal of the protein. The domain nomenclature is the same as in (Roll-Mecak \textit{et al.}, 2000). The structure is based on PDB file 1Z01 and rendered with the program PyMOL (DeLano, 2002; http://www.pymol.org)}
\end{figure}
active complexes (Milon et al., 2008), we defined \( k_0 \) as the inverse of the time, \( t_{0.5} \), at which 50% of the 70S initiation complexes have been formed after mixing (see Experimental procedures). The initiation rate, \( k_0 \), was lowest for wild-type IF2 (1.3 s\(^{-1}\)) and highest for the A1 mutant (1.8 s\(^{-1}\)) (Fig. 4B). It varied but little with increasing initiator tRNA concentration above 1 \( \mu \)M (Fig. 4C–E, Fig. S1). Lineweaver–Burk (L-B) plots of the dependence of the initiation rate, \( k_0 \), on the inverse of Met-tRNA concentration (Fig. 4F) show that the minimal initiation time obtained by extrapolation to saturating fMet-tRNA concentration varied from 0.18 s for A1 IF2 mutant up to 0.24 s for wild-type IF2. These minimal times correspond to maximal initiation rates of 5.5 and 4.1 s\(^{-1}\) for the A1 mutant and wild-type IF2 respectively. Note that higher IF3 concentration in experiments in Fig. 4A (1 \( \mu \)M IF3) than in Fig. 4C–E (0.5 \( \mu \)M IF3) accounts for a slower initiation in the former case (Antoun et al., 2006a).

**Initiation with IF2 mutants and un-formylated Met-tRNA.**

The rates of initiation in experiments where 50S subunits and un-formylated Met-tRNA were mixed with tRNA-lacking 30S PICs (Fig. 5A) were significantly lower than the initiation rates with formylated fMet-tRNA (compare Fig. 4B with Fig. 5B). In addition, the rates obtained with Met-tRNA, displayed much larger relative differences between the different IF2 variants, with an almost three-fold larger initiation rate for the A1 IF2 mutant than for wild-type IF2 (Fig. 5B).

Initiation with Met-tRNA also depended strongly on the initiator tRNA concentration for all IF2 variants (Fig. 5C–E, Fig. S2), in contrast to initiation with fMet-tRNA (compare Fig. 4F with Fig. 5F). The slow Met-tRNA binding to the 30S PIC at low Met-tRNA concentration is seen in Fig. 5 as a pronounced time-delay in the formation of 70S initiation complexes after the start of the initiation reaction.

The L-B plots (Fig. 5F) of the dependence of the initiation time, \( 1/k_0 \), on the inverse of the Met-tRNA concentration determine the sensitivity (\( k_{\text{max}}/K_M \)) of the initiation rate, \( k_0 \), to the Met-tRNA concentration and the maximal initiation rate in the limit of saturating Met-tRNA concentration (\( k_{\text{max}} \)). In the presence of either one of the A-IF2 or B-IF2 mutants the \( k_{\text{max}} \)-values were around 4 or 2 s\(^{-1}\), respectively, whereas with wild-type IF2, \( k_{\text{max}} \) was about 1 s\(^{-1}\) (Table 2). Although both \( k_{\text{max}}/K_M \) and \( k_{\text{max}} \) were increased by the IF2 mutations, the increase was larger in \( k_{\text{max}} \) than in \( k_{\text{max}}/K_M \) (Table 2), showing that the increased rate of 50S docking to the 30S subunit was the predominant effect of these mutations (see also Supporting information).

The L-B plots also show how the initiation time, \( 1/k_0 \), varied for different IF2s at a fixed concentration of Met-tRNA (Fig. 5F). Remarkably, when the initiation time was plotted against the generation time of fmt-deficient strains harbouring these IF2 mutations, a strong linear correlation was observed (Fig. 6). The correlation was robust to changes in Met-tRNA concentration in the 1–2 \( \mu \)M range (Fig. S3), corresponding to the estimated in vivo range for the free concentration of initiator tRNA (Gualerzi and Pon, 1990). Notably, the time of about 0.25 s for in vitro initiation with fMet-tRNA (Fig. 4F) and the generation time for the wild-type strain (25.2 min in Fig. 6) were on the same straight line as the mutant points (see Discussion).

**IF2 mutants cause aberrant 70S initiation complex formation**

*In vivo*, a formylation-proficient strain harbouring any one of the class A IF2 mutants grew slower than wild-type under all tested growth conditions (Fig. 3). In contrast, *in vitro* initiation with authentic formylated initiator tRNA

![Graph](http://example.com/graph.png)

**Table 2.** Kinetic parameters for 70S initiation complex formation upon addition of 50S subunits and Met-tRNA to tRNA-lacking 30S PICs.

| IF2 mutant | \( k_{\text{max}} \) | \( k_{\text{max}}/K_M \) | \( K_M \) |
|------------|-----------------|-----------------|--------|
| WT         | 1.01 ± 0.03     | 0.55 ± 0.02     | 1.82 ± 0.06 |
| B1         | 1.95 ± 0.12     | 0.90 ± 0.05     | 2.17 ± 0.18 |
| B2         | 1.96 ± 0.18     | 0.61 ± 0.03     | 3.21 ± 0.36 |
| B3         | 1.74 ± 0.09     | 0.83 ± 0.05     | 2.09 ± 0.15 |
| A1         | 3.89 ± 0.31     | 1.59 ± 0.11     | 2.44 ± 0.22 |
| A2         | 3.87 ± 0.21     | 1.83 ± 0.09     | 2.12 ± 0.16 |
| A3         | 4.06 ± 0.12     | 1.31 ± 0.03     | 3.11 ± 0.09 |
proceeded faster with class A IF2 mutants than with wild-type IF2 (Fig. 4B and 4F), which suggested that the growth rate reduction with class A IF2 mutants in vivo had other reasons than impaired mainstream initiation.

To identify these reasons, we first studied the formation of an ‘abortive’ 70S initiation complex (i.e. a complex unable to provide the donor in peptidyl-transfer) after rapid mixing of 50S subunits plus deacylated tRNAi with tRNA-lacking 30S PICs (Fig. 7A). At a final tRNAi concentration of 2 mM, the rate of abortive 70S initiation complex formation was 0.15 s⁻¹ for wild-type, approximately 0.35 s⁻¹ for B- and 0.75 s⁻¹ for the class A IF2 mutants (Fig. 7B).

Next, we studied the rate of formation of aberrant 70S initiation complexes, containing the aminoacylated form of the elongator tRNAphe in the presence of wild-type IF2 or the A1 mutant. With mRNA where the initiation codon was optimally positioned in relation to the Shine–Dalgarno sequence, the rate of aberrant 70S complex formation with Phe-tRNAphe was fivefold higher in the presence of the A1 mutant than the wild-type IF2 (Fig. 7C and D). Taking into account that the formation of 70S initiation complex with fMet-tRNAi proceeded similarly for all IF2s (Fig. 4F), the experiments in Fig. 7 suggest that, at a given free concentration of deacylated tRNAi or acylated elongator tRNA in the cell, the class A

Fig. 4. Kinetics of 70S initiation complex formation after rapid mixing of tRNA-free 30S PICs containing different IF2s with 50S subunits and fMet-tRNAi, and their dependence on fMet-tRNAi concentration.

A. 30S PICs assembled with 1 mM IF3 were mixed with 50S subunits and 0.5 mM fMet-tRNAi.

B. Rates (k) of 70S initiation complex formation in the experiments shown in (A).

C. 30S PICs assembled with 0.5 mM IF3 were mixed with 50S subunits and fMet-tRNAi, in 1, 2, 4 or 6 mM concentration.

D. The same as (C) but 30S complexes contained B2 IF2 mutant.

E. The same as (C) but 30S complexes contained A1 IF2 mutant.

F. L-B plot of the dependence of initiation time t₀.5 (= 1/k) on the inverse of fMet-tRNAi concentration for different IF2 variants.

All concentrations in the figures are given as final concentrations after the mixing.
mutants of IF2 would cause a fivefold higher frequency of formation of abortive or aberrant 70S initiation complex.

Figure 7C and D also shows that swapping of the Phe and initiation codons in mRNA, placing the former in the optimal position in relation to the Shine–Dalgarno sequence, increased the rate of aberrant 70S complex formation about 50% with the A1 mutant IF2 and about threefold with the wild-type IF2, reducing the effect of the A1 mutation in IF2 on initiation with Phe-tRNAPhe (or deacylated tRNAPhe) to about threefold (Fig. 7C and D).

The A1 IF2 mutation decreases the effect of tRNAPheacylation and formylation on the rate of 50S docking to the 30S pre-initiation complex

We studied the effects of tRNA acylation and formylation on the rate of 50S docking to the complete 30S PIC by rapidly mixing 50S subunits with 30S PICs already containing either fMet-tRNAPhe, Met-tRNAPhe, or deacylated tRNAPhe (Fig. 8). In this experiment, with the tRNA binding step omitted, removal of the formyl group of fMet-tRNAPhe led to a sevenfold reduction in subunit joining rate (from 9 to 1.3 s⁻¹) with wild-type IF2, whereas for the A1 mutant the
A rate reduction was less than twofold (from 10 to 6.6 s⁻¹). Removal of methionine led to a further sevenfold reduction in the subunit joining rate (from 1.3 to 0.18 s⁻¹) for wild-type IF2 and a threefold reduction for the A1 mutant IF2 (from 6.6 to 2.2 s⁻¹). These observations show that the loss of formylation and methionylation of fMet-tRNAi had a much smaller effect on subunit joining with the A1 IF2 mutant than with wild-type IF2 (5- versus 50-fold reduction in the subunit joining rate).

With regard to wild-type IF2, the sevenfold reduction in subunit joining rate due to removal of the formyl group of fMet-tRNAi observed here (Fig. 8) is much smaller than the previously reported 50-fold rate reduction (Antoun et al., 2006b). Here we have observed such a reduction only with deacylated tRNA. This suggests that the reason for the discrepancy between the present and the previous results could be a fast de-acylation of pre-charged purified Met-tRNAi used in previous experiments (Antoun et al., 2006b). In all experiments in this study the methionylation level of Met-tRNAi was kept high by the presence of Met, MetRS and ATP in the reaction mixtures.

Discussion

Disruption of the fmt gene in eubacteria results in a four- to 10-fold reduction in growth rate due to inefficient initiation of protein synthesis with non-formylated initiator tRNA (Guillon et al., 1992; 1996; Steiner-Mosonyi et al., 2004; Nilsson et al., 2006). This formylation deficiency can, however, be compensated by several types of mechanisms, including an increase in Met-tRNAi concentration by amplification of the metZ and metW genes, encoding tRNAi, (Nilsson et al., 2006), an increase in IF2

Fig. 6. Correlation between generation times of fmt mutant strains harbouring different IF2s and wild-type and the in vitro initiation times measured with 1 μM Met-tRNA added together with 50S subunits to tRNA-free 30S PIC containing corresponding IF2s.
concentration (Guillon et al., 1996) or an increase in IF2 affinity to Met-tRNA by mutations in the initiator tRNA binding domain IV of IF2 (Steiner-Mosonyi et al., 2004). Here, we isolated several fast-growing mutants with amino acid substitutions in IF2 that compensated for the lack of the FMT enzyme. These different mutations increased the growth rate by two- to threefold relative to that observed with wild-type IF2. Differently to what was observed for previously isolated compensatory mutations in IF2 (Steiner-Mosonyi et al., 2004), none of the mutations found here were located in the fMet-tRNA binding domain IV of IF2, which excluded any direct effect of these mutations on IF2 affinity to Met-tRNA. Instead, most mutations (8 of 13) were found in domain III of IF2, two in the linker region of IF2 connecting domains III and IV, two in the G domain and one in the N-terminal domain of IF2 (Table 1, Fig. 2). The mutations could be roughly separated into two classes (A and B) according to the extent of their compensatory effect on the growth rate in the fmt background (Fig. 1). Importantly, we found that the fitness cost in formylation-proficient strains was most severe for the class A mutations in IF2 that showed the strongest compensatory effect (Fig. 3).

**Effects of IF2 mutations on in vitro initiation account for their in vivo phenotypes**

During initiation of bacterial protein synthesis, IF2 plays pivotal roles in the binding of fMet-tRNA, to the 30S pre-initiation complex and in the subsequent fast docking of the 50S subunit to the 30S PIC containing fMet-tRNA (Benne et al., 1973; Wintermeyer and Gualerzi, 1983; Antoun et al., 2003; Antoun et al., 2006a; Grigoriadou et al., 2007). Biochemically, these two steps were jointly studied here in experiments where 50S subunits and fMet-tRNA, or Met-tRNA, were rapidly mixed with tRNA, lacking 30S PICs in a stopped-flow instrument to monitor 70S initiation complex formation by light scattering (Antoun et al., 2004).

Initiation with fMet-tRNA, occurred with similar rate, $k$, for all mutant and wild-type IF2s (Fig. 4). The rate varied little with the fMet-tRNA, concentration (Fig. 4D), suggesting that subunit docking and not tRNA, binding was the rate limiting step, in line with previous results with wild-type IF2 (Antoun et al., 2006a). However, initiation with Met-tRNA, occurred with much lower, and Met-tRNA, concentration-sensitive, $k$-values for the A- and B-mutant IF2 classes and wild-type IF2 (Fig. 5, Table 2). Such a strong dependence of the in vitro initiation rate on Met-tRNA, concentration also for wild-type IF2 (Fig. 5) explains why the amplification of initiator tRNA genes has a strong compensatory effect on the growth rate of fmt-deficient strains harbouring wild-type IF2 (Nilsson et al., 2006).

The initiation rate, $k$, approached $k_{\text{max}}$-values of 1, 2 and $4 \text{s}^{-1}$ for wild-type, class B and class A IF2 mutants, respectively, at saturating concentration of Met-tRNA, (Table 2), showing that the subunit joining rate in the presence of non-formylated Met-tRNA, was twofold faster for class B and fourfold faster for class A IF2 mutants than for wild-type IF2. In addition, there was a strong linear correlation between the in vitro initiation time, $1/k$, with Met-tRNA, and the generation time of the formylation-deficient strains harbouring the corresponding mutant and wild-type IF2s (Fig. 6). The linear correlation also included the initiation and generation times for wild-type IF2 with fMet-tRNA. Thus, the initiation time measured in vitro and the cell generation time displayed a linear dependence in a broad range of generation times (Fig. 6), and we concluded that the enhanced initiation efficiency with Met-tRNA, for the IF2 mutants as compared with wild-type IF2 accounts for the growth compensatory effects of these mutations under formylation-deficient conditions.

**Fitness cost and formation of aberrant 70S complexes with class A IF2 mutants**

In vitro initiation with fMet-tRNA, was faster with the class A IF2 mutants than with wild-type IF2 (Fig. 4F). In contrast, formylation-proficient strains with class A mutations...
in IF2 grew more slowly than wild-type strain (Fig. 3). Our in vitro experiments suggest that the explanation for the reduction in growth rate of the class A IF2 mutants is an increased frequency of aberrant initiation events.

In vitro initiation with deacylated tRNA, and class A or class B IF2 mutants was about fivefold or twofold faster, respectively, than initiation with wild-type IF2 (Fig. 7B). Furthermore, in vitro initiation with the acylated elongator Phe-tRNAfMet was about fivefold faster with the A1 mutant than with the wild-type IF2 (Fig. 7D). Since the initiation rates with wild-type and mutant IF2s were similar in the presence of fMet-tRNAiMet (Fig. 4F), these results suggest a higher frequency of aberrant initiation events in the strains harbouring class A IF2 mutants than in IF2 wild-type strain. Aberrant 70S complexes containing deacylated tRNA can be efficiently recycled back to the 30S and 50S subunits by the joint action of ribosomal recycling factor RRF and elongation factor EF-G (Pavlov et al., 2008). In contrast, aberrant 70S complexes with acylated elongator tRNAs are resistant to recycling and will participate in protein elongation, leading to out-of-frame initiation and the appearance of erroneous and potentially harmful intracellular proteins (Hirokawa et al., 2004; Pavlov et al., 2008). Elevated levels of aberrant initiation events with acylated elongator tRNAs in the formylation-proficient background in bacteria harbouring class A IF2 mutations may therefore be the primary reason for the significant fitness reduction associated with these mutants (Fig. 3).

The conformation of mutant IF2s on the 30S subunit
The rate of subunit docking with fMet-tRNAi in the 30S PIC was similar for wild-type and A1 mutant IF2 (Fig. 8). When, however, deacylated tRNA, replaced fMet-tRNAi, this rate dropped down 50-fold for wild-type IF2 but only fivefold for the A1 mutant IF2 (Fig. 8). Also, in the absence of any tRNA in the P site of the 30S PIC, the slow subunit docking was noticeably more efficient with the A1 mutant than with wild-type IF2 (Fig. 4A). Accordingly, the A1 mutation in IF2 made the rate of docking of the 50S subunit to the pre-initiation 30S complex much less dependent on formylation/methionylation of tRNA, or even the presence of the initiator tRNA. Thus, we speculate that the class A mutations in the domain III of IF2 led to a higher propensity of 30S bound IF2 to acquire its 50S docking conformation (Antoun et al., 2006a; Simonetti et al., 2008) even in the absence of tRNA. Addition of deacylated tRNA, or Met-tRNAi, led to successively higher stabilization of the 50S docking conformation for mutant and wild-type IF2, with the former at an advantage due to its intrinsic propensity for the docking conformation. In the presence of the authentic initiator tRNA, fMet-tRNAi, both A1 mutant and wild-type IF2 had almost completely switched conformation to the docking prone form, and hence the rate of subunit docking with fMet-tRNA, was much more similar for these two IF2 variants (Fig. 8).

Evidence for mRNA-limited protein synthesis
The initiation times (equal to the inverse of the effective initiation rates, $1/k_i$), estimated from our biochemical experiments for the different IF2 variants (Fig. 5F), correlate linearly with the generation times (equal to the inverse of the growth rates) of the corresponding bacterial strains (Fig. 6). The shortest initiation time (0.25 s), obtained for fMet-tRNA, and wild-type IF2 (Fig. 4F), corresponds to the shortest generation time (25.2 min) of a formylation-proficient strain with wild-type IF2. The longest initiation time (2.8 s), obtained for Met-tRNA, and wild-type IF2, corresponds to the longest generation time (101 min) of a formylation-deficient strain with wild-type IF2. How can a 2.5 s increase in initiation time result in a fourfold increase in generation time (Fig. 6)? The explanation, we propose, is mRNA-limited protein synthesis. This is a physiological state in which the total rate of protein synthesis per cell volume, $V_p$, is insensitive to the total ribosome concentration and proportional to the total mRNA concentration, [mRNA] (see Experimental procedure for details), where $n_c$ is the average number of codons per mRNA and $t_{reg}$ is the time the 30S subunit binding to the mRNA for 70S initiation complex formation ($t_{70S}$) plus the time ($t_{clear}$) for ribosomal translation in the open reading frame far enough to allow for the binding the next 30S subunit to the same mRNA. Since $V_p$ is proportional to the bacterial growth rate (Ehrenberg and Kurland, 1984), the generation time is directly proportional to $t_{reg} = t_{70S} + t_{clear}$ under mRNA-limited conditions.

The shortest possible distance between ribosomes observed in tightly packed bacterial polysomes is about 72 nucleotides (nts) (Brandt et al., 2009) which, at an elongation rate of 20 aminoacids per second (60 nt s$^{-1}$), corresponds to $t_{clear} = 1.2$ s (= 72 nts/60 nt s$^{-1}$). Our biochemical experiments show that the time, $t_{70S}$, for formation of a 70S initiation complex with fMet-tRNA, and wild-type IF2 was 0.25 s. Adding these times, one obtains a minimal time, $t_{reg}$, of 1.45 s between initiation events on mRNA in a wild-type cell with a generation time of 25 min, corresponding to an average ribosome-to-ribosome distance, $D$, on mRNA of 87 nts (obtained as $D = 1.45$ s $\times$ 60 nt s$^{-1}$). In the case of non-formylated Met-tRNA and wild-type IF2, 70S initiation complex formation proceeded slowly with $t_{70S} = 2.8$ s (Fig. 6). In this case $t_{reg} = t_{70S} + t_{clear} = 2.8 + 1.2 = 4$ s, which corresponds to an expected ribosome-to-ribosome distance on mRNA in vivo of 240 nts. The approximately threefold increase in $t_{reg}$ (4 s/1.45 s) due to the lack of Met-tRNA formylation is
close to the fourfold increase in generation time observed in vivo (Fig. 6). Furthermore, a greatly reduced ribosome density in polysomes (by a factor of 240/87) may have increased the accessibility of mRNA to ribonucleases and, hence, increased the bulk mRNA degradation rate (Arnold et al., 1998; Regnier and Arraiano, 2000; Sunohara et al., 2004), amplifying the primary effect of prolonged initiation times on in vivo generation time. Thus, under the assumption that bulk protein synthesis is mRNA-limited, our biochemical data account for the generation times measured in vivo.

The weight fraction of mRNA per total RNA at moderate growth rates was estimated to be about 2.5%, while the fraction of ribosomal RNA (rRNA) plus transfer RNA (tRNA) was about 98% (Baracchini and Bremer, 1987). From these numbers a ribosome-to-ribosome distance, D, in polysomes was estimated as 115 nts at a generation time (t₉) of 45 min (Baracchini and Bremer, 1987). More refined estimates for this distance at fast growth rates obtained recently by a similar method (Bremer and Dennis, 2008) show that D = 88 nts at t₉ = 24 min and D = 69 nts at t₉ = 20 min. The D-value of 88 nts corresponds well with our model estimate D = 87 nts for t₉ = 25 min (see above). Importantly, the distance of 69 nts is below the D-limit of 72 nts for the tightest ribosome packing in polysomes (Brandt et al., 2009), indicating that at the highest growth rate there is not enough mRNA in the cell to accommodate all ribosomes and protein synthesis is therefore mRNA-limited. This implies that in our experiments the wild-type strain was growing at or near mRNA-limited conditions, while the various mutants were growing under strict mRNA-limited conditions. From this interpretation follows the prediction that the formylation-deficient strains with impaired initiation had larger fractions of free 70S ribosomes and ribosomal subunits than the wild-type formylation-proficient strain, as previously found for strains with reduced IF2 concentration (Cole et al., 1987).

**Development of antibiotic resistance**

Finally, the presented data are highly relevant for the question of antibiotic resistance development. Antibiotic resistance is usually associated with reduced fitness (Andersson and Levin, 1999; Andersson, 2006) and at a given antibiotic pressure this fitness cost is a main determinant of the rate of development as well as the steady-state level of resistance (Levin et al., 1997; Levin, 2002). The association of resistance with decreased fitness suggests that a reduction in the use of antibiotics would lead to a reduction in the frequency of resistant bacteria by natural selection. However, the fitness cost of resistance can be reduced at unaltered resistance by additional second-site compensatory mutations (Andersson and Levin, 1999; Andersson, 2006). The fitness costs associated with fmt mutations that cause resistance to PDFIs can be reduced by increased tRNA expression via gene amplification of the metZW genes (Nilsson et al., 2006) or by point mutations in IF2 (this work). This multitude of different compensatory pathways suggests that the fitness costs of fmt mutations conferring resistance to PDFIs can be rapidly reduced by mutations, increasing both the rate of development and the steady-state level of PDFI resistance in clinical settings. A deeper understanding of the mechanisms by which bacteria reduce the fitness cost associated with drug resistance helps in the choice and development of drugs and drug targets for which adaptation is slow.

**Experimental procedures**

**Strains**

For all experiments, except where specifically indicated, the organism used was S. enterica serovar typhimurium LT2 (S. typhimurium). The minimal inhibitory concentrations (MICs) for the wild-type strain, the actinonin resistant fmt mutants and the fmt, infB double mutants were, 64 mg l⁻¹, >1024 mg l⁻¹ and >1024 mg l⁻¹ respectively.

**Compensatory evolution and identification of compensatory mutations**

Two slow-growing actinonin-resistant mutants with mutations in fmt were subjected to compensatory evolution. For each strain, 10–15 independent lineages were serially passaged in Luria–Bertani (LB) broth. When growth-compensated cells constituted the majority of the population (50–150 generations of growth), one compensated mutant clone from each lineage was isolated and saved at −80°C. To identify the unknown compensatory mutations the mini-Tn10 transposon insertion technique was used (see Supporting information).

**Hydroxylamine mutagenesis**

To isolate additional IF2 mutants, hydroxylamine mutagenesis of DNA inside bacteriophage P22 was performed. To this end we used the starting strain (DA2964), which has an argG1828::Tn10 (Tet⁺) marker linked to the infB gene. A high-titre (7.5 × 10¹² pfu ml⁻¹) phage lysate (P22 HT) was prepared on this strain and the DNA inside the phage was mutagenized as described previously (Hong and Ames, 1971).

The resulting mutagenized phage lysate was used to transduce a Δfmt mutant strain (DA10066) by mixing 5 μl mutagenized phage lysate with 200 μl of an overnight bacterial culture and incubating for 1 h at 37°C. The mixture was then plated on LA supplemented with 30 mg l⁻¹ tetracycline and the plates were incubated for 48 h at 37°C. Fast growers were picked, re-streaked on tetracycline and purified on EBU plates. The picked clones were then confirmed to be P22...
sensitive and phage free. Nine individual fast growers were isolated and their \textit{infB} gene sequence.

Fitness of mutants

Fitness of the \textit{infB} mutants was measured in both \textit{\Delta fmt} and wild-type genetic backgrounds. Using a phage lysate grown on strain DA2964, an \textit{argG1828::Tn10} insertion genetically linked to \textit{infB} was transduced into the different \textit{infB} mutant strains. Subsequently, the different \textit{infB} mutants were introduced by P22 transduction into strains DA6192 (wild-type) and DA10066 (\textit{\Delta fmt}). Presence of the correct \textit{infB} mutations was confirmed by sequencing.

Complementation study

For complementation studies we prepared pBAD30::mut\textit{infBHIS} plasmids for each mutant IF2 as described in Supporting Information. The same plasmids were also used for over-production of IF2 mutants used in the \textit{in vitro} studies. Different pBAD30::mut\textit{infBHIS} constructs were transformed by electroporation into the \textit{\Delta fmt} mutant (DA10066). Fitness of all complemented mutants was estimated by measuring growth rates in rich medium. The bacteria were pre-grown to saturation in LB overnight. Approximately 10^6 cells were inoculated into 0.4 ml of fresh LB in a Bioscreen plate and the absorbance at 600 nm was read with a BioscreenC (Oy Growth Curves Ab Ltd). For each strain, growth rates were measured in quadruplicates in at least two separate experiments and relative growth rates were calculated as the growth rate of the parental strain divided by the growth rate of the tested strain.

Chemicals and buffers for \textit{in vitro} experiments

Phosphoenolpyruvate (PEP), myokinase (MK), pyruvate kinase (PK), inorganic pyrophosphatase (PPi), putrescine and spermidine were from Sigma (USA). Experiments were conducted in a polymin-like buffer, LS4, containing 95 mM KCl, 3 mM NH₄Cl, 0.5 mM CaCl₂, 8 mM putrescine, 1 mM spermidine, 30 mM HEPES pH 7.5, 1 mM DTE, 2 mM PEP, 1 mM GTP, 1 mM ATP and 5 mM Mg(OAc)₂, supplemented with 1 µM ml⁻¹ PK and 0.1 µM ml⁻¹ MK (Jelenc and Kurland, 1979; Pavlov et al., 2008). Since each ATP or GTP molecule chelates one Mg²⁺ cation, the free Mg²⁺ concentration in the LS4 buffer was adjusted to 4 mM by adding 1 mM Mg(OAc)₂.

Components of the \textit{in vitro} translation system

70S ribosomes, 50S and 30S subunits, [²⁵³H]Met-tRNA, initiation factors as well as Met and Phe anamycyl-tRNA synthetases (MetRS and PheRS) were prepared from \textit{Escherichia coli} as described in Freistroffer \textit{et al.} (1997), Antoun \textit{et al.} (2004) and Antoun \textit{et al.} (2006a). Overproduced N-terminus-His-tagged wild-type and mutant \textit{S. typhimurium} IF2s were isolated from \textit{S. typhimurium} essentially as described in Antoun \textit{et al.} (2004). Initiation tRNA and tRNA^{\text{Ac}} were from Sigma (USA), mMFTI mRNA and mMFTI mRNA with strong SD sequences were prepared as described in Pavlov \textit{et al.} (2008).

Comparison of the amino acid sequences of wild-type IF2 from \textit{S. typhimurium} and \textit{E. coli} showed that they were > 96% identical. The identity level was even higher, > 98%, when the functionally dispensable N-terminal domain of IF2 (Caserta \textit{et al.}, 2006) was excluded. Accordingly, wild-type IF2s from \textit{S. typhimurium} and \textit{E. coli} behaved practically identically in all \textit{in vitro} initiation experiments (data not shown), which justifies the use of the well-characterized \textit{E. coli} components in the biochemical experiments conducted in this study.

\textbf{In vitro kinetic experiments}

Two mixtures, 1 and 2, were prepared in the LS buffer. Mixture 1 contained 30S pre-initiation complexes assembled by mixing 0.32 µM 30S subunits, 0.8 µM mMFTI mRNA with a strong SD sequence (Pavlov \textit{et al.}, 2008), 1 µM initiation factor IF1, 0.6 µM IF2 and 0.5 µM IF3 unless specified otherwise. Mixture 2 contained 0.36 µM 50S subunits. The type and final concentration of tRNA added either to mixture 1 or mixture 2 is indicated for each experiment in the corresponding figure legend. Both mixtures (1 and 2) were pre-incubated for 20 min at 37°C. Met-tRNA, was methionylated \textit{in situ} as follows. First, a mixture containing 200 µM initiator tRNA, 1 mM [²⁵³H]Met aminocaid and 800 unit ml⁻¹ MetRS in LS4 buffer supplemented with 1 µg ml⁻¹ PK, 0.1 µg ml⁻¹ PPi and 0.1 µg ml⁻¹ MK (Jelenc and Kurland, 1979) was assembled and pre-incubated for 20 min at 37°C after which it was put on ice. Just before loading into a stopped flow instrument (see below), a portion of this mixture was added to mixture 2 containing 50S subunits (or to mixture 1 containing 30S subunits) to obtain the final Met-tRNA, concentration specified for each experiment. Phe-tRNA^{\text{Ac}} was prepared in the same way except that the acylation mixture was assembled with tRNA^{\text{Ac}}, Phe aminocaid and PheRS instead of tRNA, Met amino acid and MetRS. Pre-incubated mixtures 1 and 2 (0.6–0.8 ml volume of each) were loaded into the syringes of a stopped flow instrument (SX-20, Applied Photophysics, Leatherhead, UK). The kinetics of 70S complex formation was monitored at 37°C with light scattering after rapid mixing equal volumes (usually 0.06 ml) of mixtures 1 and 2 as described (Antoun \textit{et al.}, 2006a). All concentrations in mixes 1 and 2 specified above are the final concentrations after the mixing.

\textbf{Treatment of light scattering data}

Each light scattering experiment provided 6–8 scattering traces. Those were used to obtain an average scattering trace and to estimate the average and standard deviation of the time, \(t_{0.5}\), at which a light scattering trace reached 50% of its plateau value. The time \(t_{0.5}\) obtained for the average trace was always very close to the average \(t_{0.5}\) time. The rate, \(k_5\), of the initiation reaction was defined as the inverse of \(t_{0.5}\) for the average trace as motivated by the following relation between scattering intensity and time:

\[
\frac{I(t) - I(0)}{I(\infty) - I(0)} = \frac{k_5 \cdot t}{1 + k_5 \cdot t} = \frac{k_5 \langle 50S_0 \rangle \cdot t}{1 + k_5 \langle 50S_0 \rangle \cdot t} \tag{1}
\]
This relation describes the irreversible formation of a binary complex, A:B, from particles A and B mixed at equal concentrations (Antoun et al., 2006a). The rate \( k \) in this relation is equal to the product of the second-order rate constant \( k_2 \) of the binding reaction by the initial concentration of B particles (50S subunits in our case). In the presence of IF3 expression (1) holds, but \( k_2 \) is now a compounded rate constant that depends on the concentrations of both IF3 and 50S subunits (Antoun et al., 2006a).

When the initiation reaction includes tRNA binding to the 30S PIC with association rate constant \( k_3 \) followed by the 50S subunit docking with rate constant \( k_4 \), then \( t_{ss} \) is approximated by (Supporting information):

\[
t_{ss} = \frac{1}{k_1 \cdot [(f)\text{Met} - \text{tRNA}]} + \frac{1}{k_2 [50S_5]}
\]  
(2)

The L-B representation of this expression is:

\[
t_{ss} = \frac{K_M}{k_{\text{max}}} \cdot \frac{1}{[(f)\text{Met} - \text{tRNA}]} + \frac{1}{k_{\text{max}}}
\]  
(3)

Comparison of (2) and (3) clarifies the relation between rate constants \( k_1 \) and \( k_2 \) and L-B parameters \( k_{\text{max}}/K_M \) and \( k_{\text{max}} \).

The scattering traces were fitted to a four-parameter kinetic model (Fig. S4B) describing tRNA binding to active 30S PICs (rate constant \( k_1 \)), subsequent docking of 50S subunits to tRNA-containing, active 30S PICs (rate constant \( k_2 \)) and a slow conversion (described by rate constant \( k_3 \) and \( k_4 \)) of a small fraction of subunit-docking-inactive 30S PICs (see Supporting information for details). For clarity of presentation we used a digital filter to reduce the noise and the numbers of data points in the scattering traces shown in the figures.

Definition of mRNA-limited protein synthesis in growing cells

The total rate of protein synthesis per cell volume can be written as (see section D in Supporting information for details):

\[
V_p = \frac{n_i}{t_{\text{reg}}} [\text{mRNA}]_0 \cdot \frac{\tau_{\text{reg}} k_3 [30S]}{1 + \tau_{\text{reg}} k_3 [30S]}
\]  
(4)

Here, [mRNA]_0 is the total mRNA concentration in the cell, \( n_i \) is the average number of codons per mRNA, [30S] is the concentration of free 30S subunits and \( k_3 \) is the rate constant for 30S subunit association to mRNA. The time \( t_{\text{reg}} = \tau_{\text{reg}} + \tau_{\text{clear}} \) is the time after 30S subunit binding to the mRNA for 70S initiation complex formation \( (\tau_{\text{reg}}) \) plus the time \( (\tau_{\text{clear}}) \) for ribosomal translation of the mRNA ORF far enough to allow for the binding of a next 30S subunit to the mRNA. The quadratic equation:

\[
[30S_5] = [30S] + [\text{mRNA}]_0 \cdot \frac{(n_i/\nu_e + \tau_{\text{reg}}) k_3 [30S]}{1 + \tau_{\text{reg}} k_3 [30S]}
\]  
(5)

relates [30S] to the total concentration, [30S], of the 30S subunit (equal to the total ribosome concentration) and [mRNA]_0. Here, \( \nu_e \) is the elongation rate of translating ribosome in codons per second. Together, relations (4) and (5) determine how \( V_p \) depends on [mRNA], [30S] for any choice of the parameters \( \tau_{\text{reg}}, \tau_{\text{clear}}, k_3, n_i, \nu_e \) as exemplified in Fig. S5.

In the limiting case, where \( \tau_{\text{reg}} k_3 [30S] >> 1 \), relation (4) is approximated by:

\[
V_p = \frac{[\text{mRNA}]_0}{\nu_e} \cdot \frac{n_i}{\tau_{\text{reg}}}
\]  
(6)

This defines the condition of mRNA limitation, where \( V_p \) is proportional to [mRNA]_0 and inversely proportional to \( \tau_{\text{reg}} \). In this limit, relation (5) is approximated by:

\[
[30S] = [30S] - [\text{mRNA}]_0 \frac{(n_i/\nu_e + \tau_{\text{reg}})}{\tau_{\text{reg}}}
\]  
(7)

The ratio \( (n_i/\nu_e + \tau_{\text{reg}})/\tau_{\text{reg}} \) in relation (7) defines the maximal number of ribosomes on an mRNA with \( n_i \) codons (see section D of the Supporting information). From relation (7) it follows that the condition \( \tau_{\text{reg}} k_3 [30S] >> 1 \) for mRNA-limited protein synthesis requires that

\[
[\text{mRNA}]_0 < \frac{\tau_{\text{reg}}}{(n_i/\nu_e + \tau_{\text{reg}}) [30S]}
\]  
(8)

When \( \tau_{\text{reg}} k_3 [30S] >> 1 \), as in the realistic cases illustrated in Fig. S5, inequality (8) approximates the region in which protein synthesis is mRNA-limited.

Acknowledgements

This work was supported by the Swedish Research Council (to ME and DIA) and the European Union 6th and 7th Framework Programmes (to DIA).

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Supporting information

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