

# Differential Effects of Thiopeptide and Orthosomycin Antibiotics on Translational GTPases

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## SUMMARY

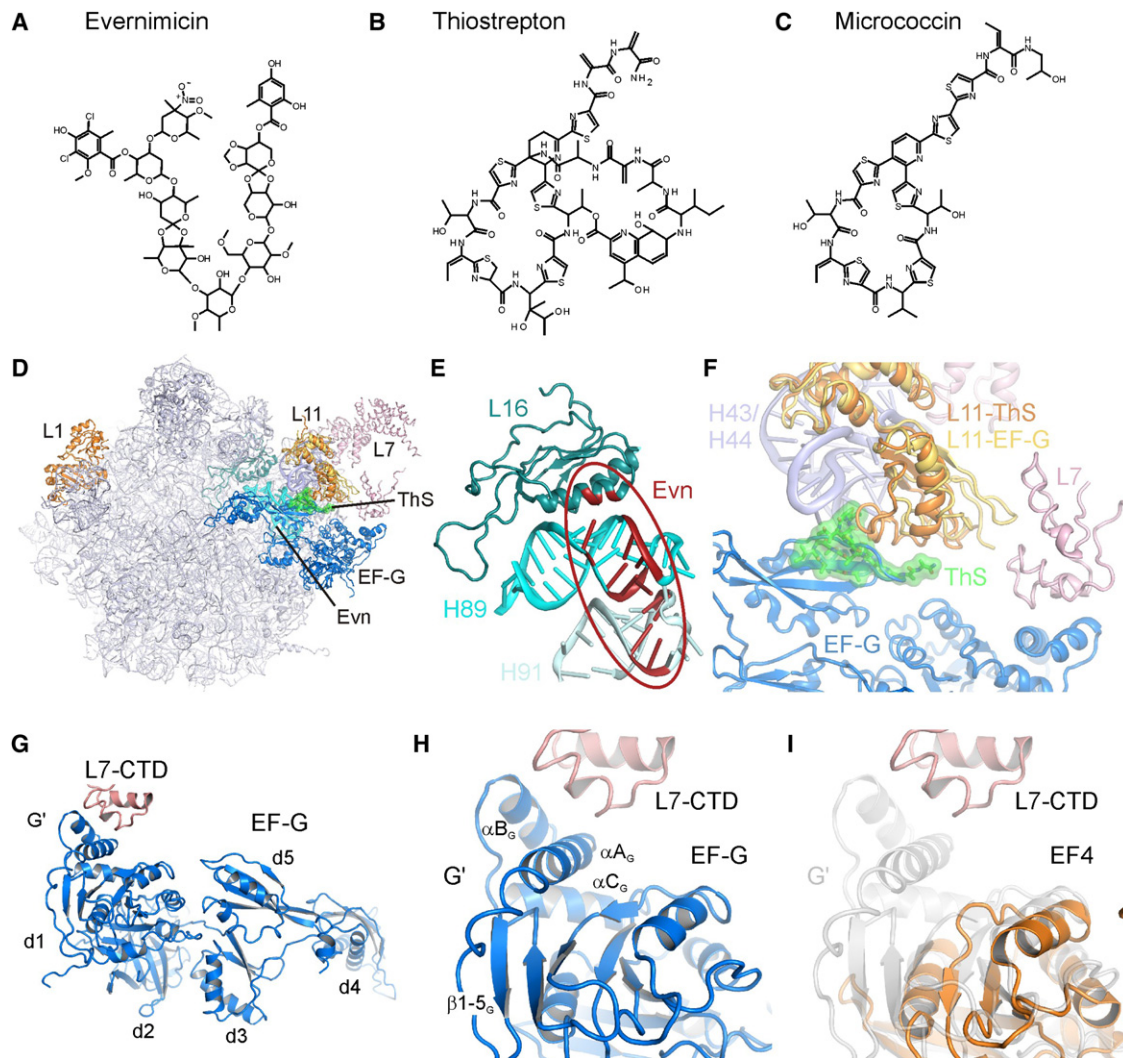
The ribosome is a major target in the bacterial cell for antibiotics. Here, we dissect the effects that the thiopeptide antibiotics thiostrepton (ThS) and micrococin (MiC) as well as the orthosomycin antibiotic evernimicin (Evn) have on translational GTPases. We demonstrate that, like ThS, MiC is a translocation inhibitor, and that the activation by MiC of the ribosome-dependent GTPase activity of EF-G is dependent on the presence of the ribosomal proteins L7/L12 as well as the G' subdomain of EF-G. In contrast, Evn does not inhibit translocation but is a potent inhibitor of back-translocation as well as IF2-dependent 70S-initiation complex formation. Collectively, these results shed insight not only into fundamental aspects of translation but also into the unappreciated specificities of these classes of translational inhibitors.

## INTRODUCTION

Protein synthesis occurs on large macromolecular particles called ribosomes, which are composed of RNA and protein. In bacteria the 70S ribosome can be split into a small (30S) and large (50S) subunit. The bacterial translational machinery represents a major target within the cell for antibiotics (reviewed by [Blanchard et al., 2010](#); [Wilson, 2009](#)). Many clinically important classes of antibiotics inhibit translation by binding to the active centers of ribosome. For example the tetracyclines and aminoglycosides bind at the decoding site on the small subunit, and the chloramphenicols, macrolides/ketolides, oxazolidinones, and lincosamides bind at the peptidyltransferase center (PTC) on the large subunit ([Sohmen et al., 2009a, 2009b](#)). Despite the potency of many of these drug classes, antibiotic resistance among clinically relevant pathogens is an increasing problem, and thus, the need for new antibiotics is more urgent than ever before. Ideally, the new antibiotics should have nonoverlapping sites with the currently used antimicrobial agents, so that the

occurrence of cross-resistance is reduced or prevented. Two such classes are the thiopeptides and orthosomycins ([Figures 1A–1C](#)), which bind to distinct sites on the large ribosomal subunit that are located far from the PTC ([Figures 1D and 1E](#)).

The orthosomycins, such as evernimicin (Evn), are oligosaccharide antibiotics ([Figure 1A](#)) that display excellent antimicrobial activity against a broad range of Gram-positive bacteria, both in vivo and in vitro. Although attempts to introduce Evn clinically as Ziracin (Schering-Plough) were unsuccessful, a related compound, avilamycin (Avn), is used as a growth promoter in animal feeding. A multitude of resistance mutation/modification and chemical footprinting studies indicate that the orthosomycin-binding site is located at the base of the L7/L12 stalk ([Figures 1D and 1E](#)), ~50 Å from the PTC. Mutations in ribosomal protein L16 ([Aarestrup and Jensen, 2000](#); [Adrian et al., 2000b](#); [McNicholas et al., 2001](#); [Zarazaga et al., 2002](#)), and in helix 89 (H89) and H91 of the 23S rRNA, as well as methylation of G2470 (*E. coli* numbering used throughout) in H89 ([Mann et al., 2001](#)), confer resistance to Evn and Avn ([Adrian et al., 2000a](#); [Belova et al., 2001](#)). In addition, Evn and Avn protect 23S rRNA nucleotides, e.g., A2482 in H89 and A2534 in H91, from chemical modification ([Belova et al., 2001](#); [Kofoed and Vester, 2002](#)). It is also noteworthy that mutations in *rpL16* (L16 gene) confer relatively low-level resistance (MIC <12 µg ml<sup>-1</sup>), whereas higher-level resistance (MIC >256 µg ml<sup>-1</sup>) is obtained by EmtA-mediated methylation or rRNA mutations ([Belova et al., 2001](#); [Mann et al., 2001](#)). Taken together, these results suggest that the orthosomycin-binding site spans from the minor groove of H89 to the loop region of H91 ([Figure 1E](#)) and that mutations in L16 confer resistance indirectly via perturbation of the 23S rRNA. In agreement with this novel location, Evn does not inhibit peptide-bond formation ([Belova et al., 2001](#)) or compete with several other ribosomal antibiotics for ribosome binding ([McNicholas et al., 2000](#)). Although some effect of Avn on aa-tRNA binding to ribosomes has been observed ([Wolf, 1973](#)), Evn is better known as an initiation inhibitor; Evn inhibits the formation of fMet-puromycin in an IF2-dependent manner ([Belova et al., 2001](#)), although the exact step of inhibition remains unclear. Moreover, to our knowledge, the effects of orthosomycins on translation factors other than IF2 and EF-Tu have not yet been addressed.



**Figure 1. Chemical Structures and Ribosomal-Binding Sites of Thiopeptide and Orthosomycin Antibiotics**

(A–C) Chemical structures of the (A) orthosomycin Evn, and the thiopeptide antibiotics (B) ThS and (C) MiC.

(D) Overview of the binding sites of orthosomycins and thiopeptides on the large subunit relative to EF-G. R-proteins L1, L11, and L7 are shown for reference.

(E) Putative binding site of orthosomycins spanning from H89 and H91 of the 23S rRNA. Residues highlighted in red have been associated biochemically with Evn or Avn (reviewed by [Wilson, 2009](#)).

(F) Binding site of ThS (green) in the cleft between H43 and H44 of the 23S rRNA and the NTD of L11 (L11-ThS) ([Harms et al., 2008](#)). The relative positions of EF-G (blue), C-terminal domain of L7/L12 (L7-CTD), and of a different conformation of L11 (L11-EF-G) are from [Gao et al. \(2009\)](#).

(G) Overview of domain arrangement of EF-G with contact between the L7-CTD and the G' domain of EF-G as observed in the 70S-EF-G crystal structure ([Gao et al., 2009](#)).

(H) Expansion of (G) highlighting the secondary structure elements of the G' subdomain.

(I) Juxtaposition of the G' subdomain of EF-G (gray transparency) with the G-domain of EF4 (orange) ([Evans et al., 2008](#)) that lacks a G' subdomain.

See also Figure S1.

In contrast, thiopeptides, such as thiostrepton (ThS), have been extensively studied (reviewed by [Bagley et al., 2005](#); [Nicolaou et al., 2009](#); [Wilson, 2009](#)). Although ThS is already in veterinary usage, its low water solubility and poor bioavailability has so far precluded its use in human medicine. Nevertheless, the thiopeptide class of antibiotics has received renewed interest in the recent years because (i) of its effectiveness against Gram-positive bacteria, in particular, methicillin-resistant *Staphylococcus aureus* (MRSA), and against the malarial parasite *Plasmodium falciparum* ([McConkey et al., 1997](#)), as well as (ii)

recent successes in the total synthesis of a number of thiopeptides (reviewed by [Hughes and Moody, 2007](#); [Nicolaou et al., 2009](#)), including among others, ThS ([Nicolaou et al., 2005a, 2005b](#)) and micrococcin (MiC) ([Lefranc and Ciufolini, 2009](#)). Thiopeptide antibiotics, such as ThS and MiC, are composed of oxazoles and thiazoles, as well as nonnatural amino acids that are linked together to form complex macrocyclic frameworks (Figures 1B and 1C).

Both ThS and MiC have been crystallized in complex with the large ribosomal subunit, revealing their binding site to be located

in a cleft formed by the N-terminal domain (NTD) of ribosomal protein L11 and H43/H44 of the 23S rRNA (Figures 1D and 1F) (Harms et al., 2008), consistent with a vast wealth of prior biochemical studies (reviewed by Wilson, 2009). This region is part of the GTPase-associated center (GAC), so named because it is involved in binding of translation factors and stimulation of their GTPase activities. Consistently, thiopeptide antibiotics have been shown to inhibit IF2-dependent 70S-initiation complex (70SIC) formation (Brandi et al., 2004; Grigoriadou et al., 2007), EF-Tu-dependent delivery of aminoacyl-tRNAs to the ribosome (Brandi et al., 2004; Gonzalez et al., 2007; Modolell et al., 1971; Otaka and Kaji, 1974), translocation of the tRNA<sub>2</sub>-mRNA complex through the ribosome (Munro et al., 2010; Pan et al., 2007; Pestka, 1970; Pestka and Brot, 1971; Rodnina et al., 1997), and stringent factor RelA-dependent synthesis of ppGpp (Cundliffe and Thompson, 1981; Knutsson Jenvert and Holmberg Schiavone, 2005). Surprisingly, however, ThS and MiC exhibit differential effects on the uncoupled ribosome-dependent EF-G GTPase activities: ThS strongly inhibits multiple-turnover GTP hydrolysis of EF-G (Pestka, 1970; Weisblum and Demohn, 1970) by preventing inorganic phosphate (Pi) release and, thus, trapping EF-G on the ribosome (Rodnina et al., 1999; Seo et al., 2006). The overlap between the ThS and EF-G binding sites on the ribosome (Figures 1D and 1F) (Harms et al., 2008) suggests that ThS stabilizes an initial binding state of EF-G (Rodnina et al., 1999; Seo et al., 2006), which has weaker affinity than a subsequently formed, accommodated state (Cameron et al., 2002; Seo et al., 2006). In contrast, MiC does not prevent Pi release (Starosta et al., 2009) and actually stimulates the multiple-turnover GTP hydrolysis activity of EF-G (Cameron et al., 2002; Cundliffe and Thompson, 1981; Lentzen et al., 2003).

The G domains of translational GTPases have a well-conserved architecture, with the exception of a region located between the G4 and G5 motifs, which, in EF-G, are termed the G' subdomain (see Figure S1 available online). In EF-G the G' subdomain consists of ~90 amino acids that form four consecutive  $\beta$  strands (2<sub>G</sub>–5<sub>G</sub>) followed by three  $\alpha$  helices (A<sub>G</sub>–C<sub>G</sub>) (Figures 1G and 1H; Figure S1). In contrast, translational GTPases, such as elongation factor EF-Tu, initiation factor IF2, the ribosome-associated stress response factor BipA (deLivron et al., 2009; deLivron and Robinson, 2008; Owens et al., 2004), and the back-translocation factor LepA (EF4) (Liu et al., 2010; Qin et al., 2006), are completely lacking the G' subdomain (Figure 1I), whereas the ribosomal protection protein TetM (Connell et al., 2003a) has a partial G' subdomain, lacking three  $\beta$  strands (3<sub>G</sub>–5<sub>G</sub>) (Figure S1). Interaction of the G' subdomain of EF-G with the C-terminal domain of ribosomal protein L7/L12 (L7-CTD) is observed structurally (Connell et al., 2007; Datta et al., 2005; Gao et al., 2009; Harms et al., 2008; Helgstrand et al., 2007) (Figures 1G and 1H) and is required for efficient GTP hydrolysis (Diaconu et al., 2005; Nechifor et al., 2007; Savelsbergh et al., 2000, 2005) and Pi release (Diaconu et al., 2005; Savelsbergh et al., 2005), leading to the suggestion that enhanced recycling of EF-G by MiC results from stabilization of this interaction (Harms et al., 2008).

Here, we show that although MiC stimulates the multiple-turnover ribosome-dependent EF-G GTPase, it inhibits the GTPase activities of other translational GTPases, such as TetM, EF4,

BipA, and IF2, which have reduced, or completely absent, G' subdomains. Furthermore, deletion of the G' subdomain from EF-G removes the stimulatory effect of MiC, as does the absence of L7/L12 on the ribosome. Despite the differential effects of MiC and ThS on EF-G GTPase, we show that MiC, like ThS, is a potent inhibitor of the EF-G catalyzed translocation process. In contrast the orthosomycin Evn, although not interfering with EF-G GTPase and translocation activities, is a potent inhibitor of the ribosome-dependent IF2 and EF4 GTPase activities, as well as of EF4-mediated back-translocation and IF2-dependent 70SIC formation. Collectively, our results delineate the specific steps of interference and reveal the differential effects that these inhibitors have on translocation factor function—an important step for the development of new, improved antimicrobial agents.

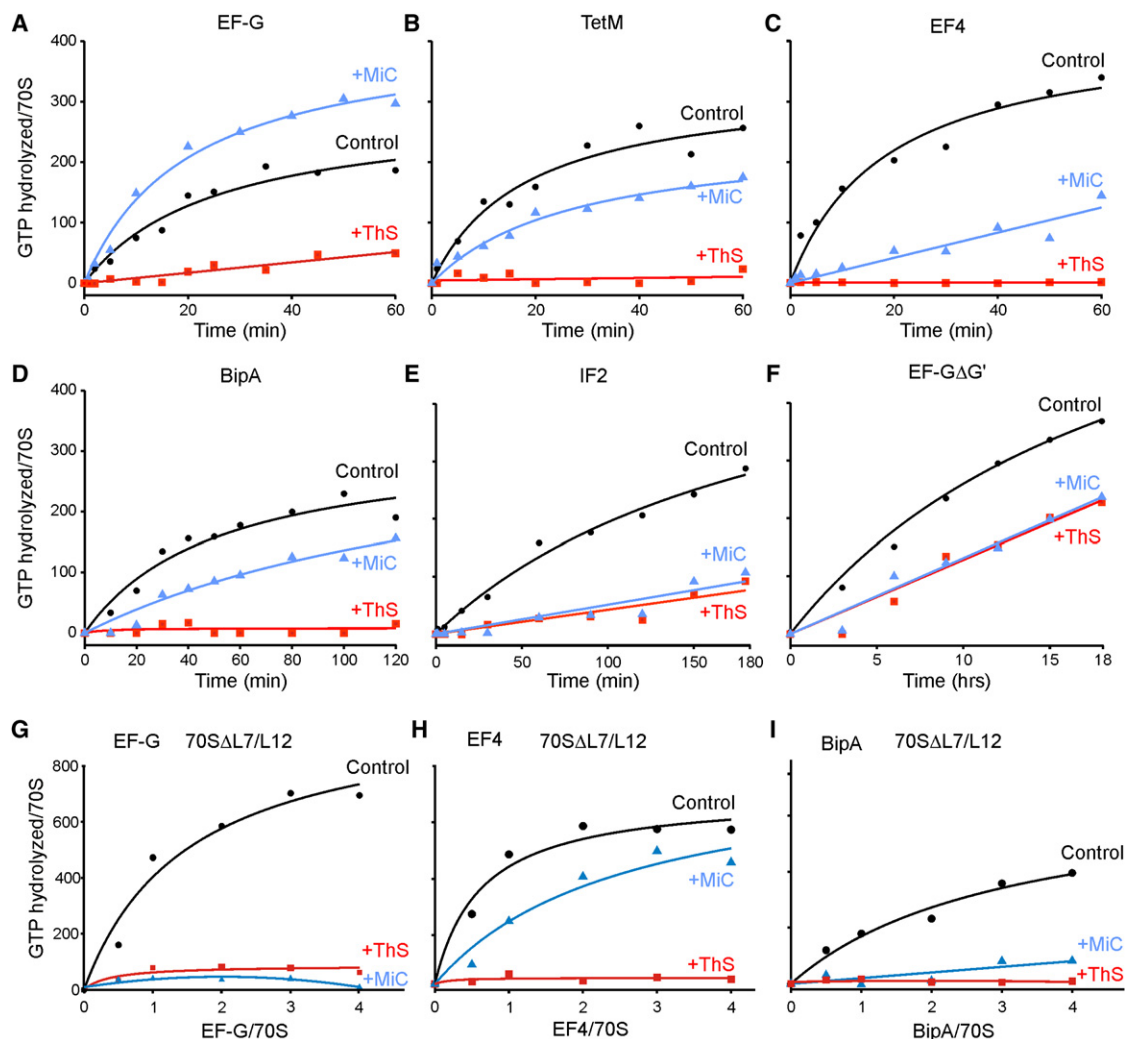
## RESULTS

### Differential Effects of Thiopeptide Antibiotics on GTPase Activities of Translational Factors

The suggestion that MiC stimulates the uncoupled ribosome-dependent GTPase (rdGTPase) of EF-G by stabilizing the interaction of L7-CTD with the G' subdomain of EF-G (Harms et al., 2008) prompted us to investigate the effect of MiC on the uncoupled rdGTPase activities of other translational GTPases that have reduced or completely absent G' subdomains (such as TetM, EF4, and BipA), as determined using the malachite green assay (Starosta et al., 2009). We found rdGTPase activity of EF-G to be inhibited by ThS (Figure 2A), as expected from previous reports (Pan et al., 2007; Pestka, 1970; Rodnina et al., 1999; Weisblum and Demohn, 1970). The rdGTPase activities of TetM, EF4, and BipA were also inhibited by ThS (1  $\mu$ M) (Figures 2B–2D), as was the rdGTPase activity of IF2 (Figure 2E), as reported previously (Grunberg-Manago et al., 1972). Similar trends were found for all factors independent of the excess of the factor over the ribosome (Figure S2) or the concentration of antibiotics used (data not shown). Consistent with these results, ThS has been shown previously to inhibit the rdGTPase activity of a related ribosome protection protein, TetO (Connell et al., 2003b), the formation of a stable complex between TetM and the ribosome in the presence of GDPNP and GTP (Dantley et al., 1998), and the IF2-dependent formation of 70SIC (Grigoriadou et al., 2007). However, we could not reproduce the recently reported stimulatory effects of ThS on IF2 GTPase (Brandi et al., 2004; Cameron et al., 2002).

Guided by a comparison of the crystal structures of *T. thermophilus* EF-G (PDB 1FNM; Laurberg et al., 2000) with *E. coli* EF4 (PDB 3CB4; Evans et al., 2008), we also generated an *E. coli* EF-G lacking the G' subdomain (EF-G $\Delta$ G'): EF-G $\Delta$ G' has a deletion of amino acids 172–265, thus truncating the G' subdomain before  $\beta$ 3<sub>G</sub> and after  $\beta$ 6<sub>1</sub> (Figure S1). In contrast to previous attempts to produce an EF-G $\Delta$ G' protein (Nechifor et al., 2007), soluble protein was obtained under native conditions, and therefore, refolding or purification under denaturing conditions was unnecessary (see Experimental Procedures). The purified *E. coli* EF-G $\Delta$ G' had an intrinsic GTPase activity comparable with that of wild-type *E. coli* EF-G (data not shown), suggesting that the protein was not misfolded. Moreover, although the rdGTPase was significantly slower (>10 $\times$ ) than wild-type





**Figure 2. Effect of ThS and MiC on GTPase Activity of Various Translation Factors**

(A–F) The rdGTPase activities of translation factors (A) *E. coli* EF-G, (B) TetM, (C) *E. coli* EF4, (D) *E. coli* BipA, (E) *E. coli* IF2, and (F) *E. coli* EF-GΔG', using *E. coli* 70S ribosomes in the absence (black circles) and presence of 1 μM ThS (red squares) or 5 μM MiC (blue triangles).

(G–I) The rdGTPase activities of *E. coli* translation factors (G) EF-G, (H) EF4, and (I) BipA, using *E. coli* 70S ribosomes lacking L7/L12. Reactions in (G)–(I) were incubated for 12 hr at 20°C.

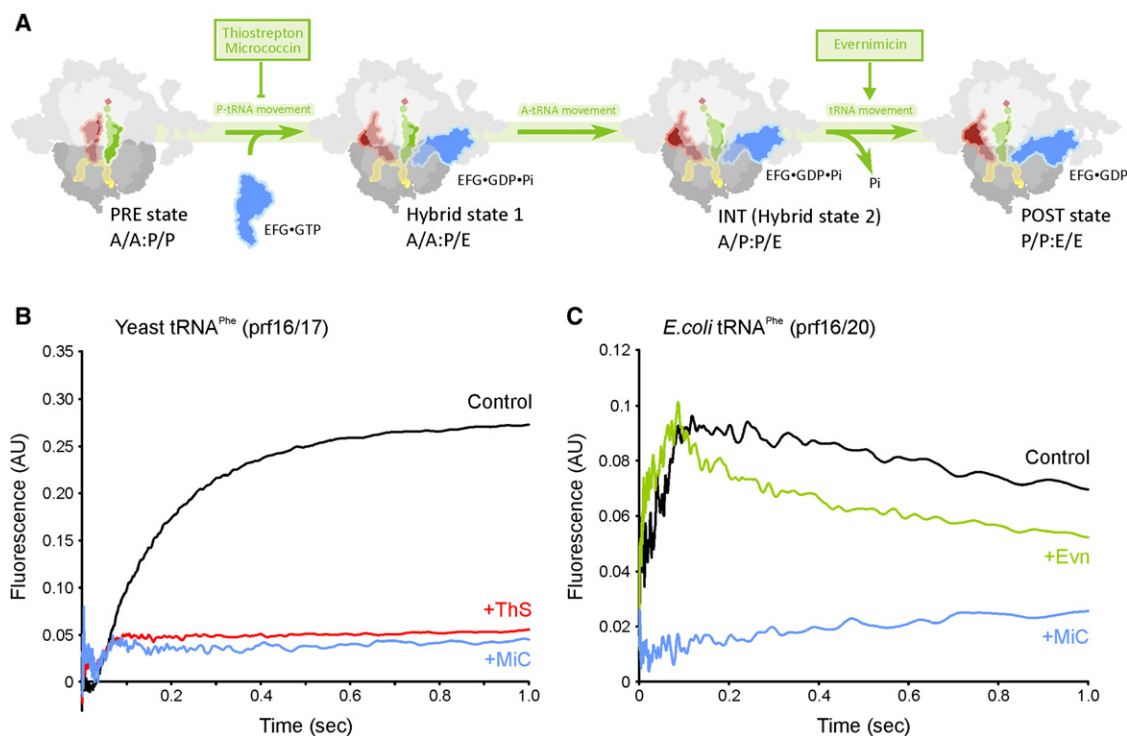
In all cases, background hydrolysis due to the intrinsic GTPase activity of each factor has been subtracted. See also Figure S2.

*E. coli* EF-G, it was nevertheless inhibited by ThS (Figure 2F), albeit more weakly than for wild-type EF-G. As expected from previous studies (Cameron et al., 2002; Cundliffe and Thompson, 1981; Lentzen et al., 2003), we also observed that MiC enhanced the rdGTPase activity of EF-G (Figure 2A). Conversely, we could show that MiC inhibited the rdGTPase of all other translational GTPases that were tested, namely TetM, EF4, BipA, and IF2 (Figures 2B–2E). Additionally, deletion of the G' subdomain of EF-G also produced a change in the activity of MiC because MiC was seen to inhibit, rather than stimulate, the rdGTPase of EF-GΔG' (Figure 2F). We note that deletion of the G' subdomain of EF-G greatly reduced (>10-fold) the rdGTPase activity of the factor, similar to the previously reported introduction of mutations within αA<sub>G</sub> of the G' subdomain (Nechifor et al., 2007). Similarly, ThS and MiC also inhibited the rdGTPase activities of EF-G, EF4, and BipA when *E. coli* 70S ribosomes were used

that lacked L7/L12 (70SΔL7/L12) (Figures 2G–2I). Defects in rdGTPase of EF-G have also been seen when the ribosomal proteins L7/L12 are selectively removed from the ribosome (Diaconu et al., 2005; Kischka et al., 1971; Nechifor et al., 2007) or mutations are made within the L7-CTD (Diaconu et al., 2005).

#### Inhibition of Translocation by Thiopeptide Antibiotics, but Not by Evn

The translocation reaction occurs after peptide-bond formation and involves the EF-G catalyzed movement of the peptidyl- and deacylated-tRNAs in the A and P sites into the P and E sites, respectively (Figure 3A) (reviewed by Schmeing and Ramakrishnan, 2009). Conversion of the pretranslocational (PRE) complex into a posttranslocational (POST) complex proceeds through (A/P and P/E) hybrid states, where the CCA 3' ends of the tRNAs move with respect to the large subunit while remaining



**Figure 3. Effect of the MiC and Evn on Translocation**

(A) Scheme for EF-G catalyzed translocation with sites of antibiotic inhibition.

(B) Isolated PRE complex (0.1  $\mu$ M) containing yeast fMetPhe-tRNA<sup>Phe</sup>(Prf16/17) in the A site and tRNA<sup>fMet</sup> in the P site, either in the absence of antibiotic (black trace) or in the presence of ThS (10  $\mu$ M; red trace) or MiC (10  $\mu$ M; blue trace), was rapidly mixed in a stopped-flow spectrophotometer with 5  $\mu$ M EF-G and 1 mM GTP.

(C) Isolated PRE complex (0.1  $\mu$ M) containing *E. coli* fMetPhe-tRNA<sup>Phe</sup>(Prf16/20) and tRNA<sup>fMet</sup> in the P site, in the absence of antibiotic (black trace) or in the presence of Evn (10  $\mu$ M; green trace) or MiC (10  $\mu$ M; blue trace), was rapidly mixed in a stopped-flow spectrophotometer with 5  $\mu$ M EF-G and 1 mM GTP. All concentrations are final after mixing. The traces in the control and +Evn traces are each fit to a two-step process (Pan et al., 2007) yielding the following rate constants: Control:  $20.7 \pm 0.6$  s<sup>-1</sup>,  $1.3 \pm 0.1$  s<sup>-1</sup>; +Evn:  $28.1 \pm 0.7$  s<sup>-1</sup>,  $3.5 \pm 0.2$  s<sup>-1</sup>.

See also Figure S3.

relatively fixed with respect to the small subunit (Blanchard et al., 2004; Moazed and Noller, 1989; Ratje et al., 2010). To monitor translocation rates, PRE complexes were assembled containing proflavin (prf)-labeled tRNAs, thus enabling tRNA movement to be followed by stopped-flow monitoring of the fluorescence change following delivery of EF-G•GTP (Pan et al., 2007; Rodnina et al., 1997; Savelsbergh et al., 2003). In Figure 3B, rapid addition of EF-G•GTP to PRE complexes formed with unlabelled *E. coli* tRNA<sup>fMet</sup> in the P site and yeast fMetPhe-tRNA<sup>Phe</sup> (prf16/17) in the A site leads to an apparent monophasic increase in fluorescence as A-site tRNA is translocated to the P site (Pan et al., 2007; Rodnina et al., 1997; Savelsbergh et al., 2003). Preincubation of the PRE complex with (10  $\mu$ M) MiC or ThS completely abolished fluorescence, as reported previously for ThS (Pan et al., 2007; Rodnina et al., 1997).

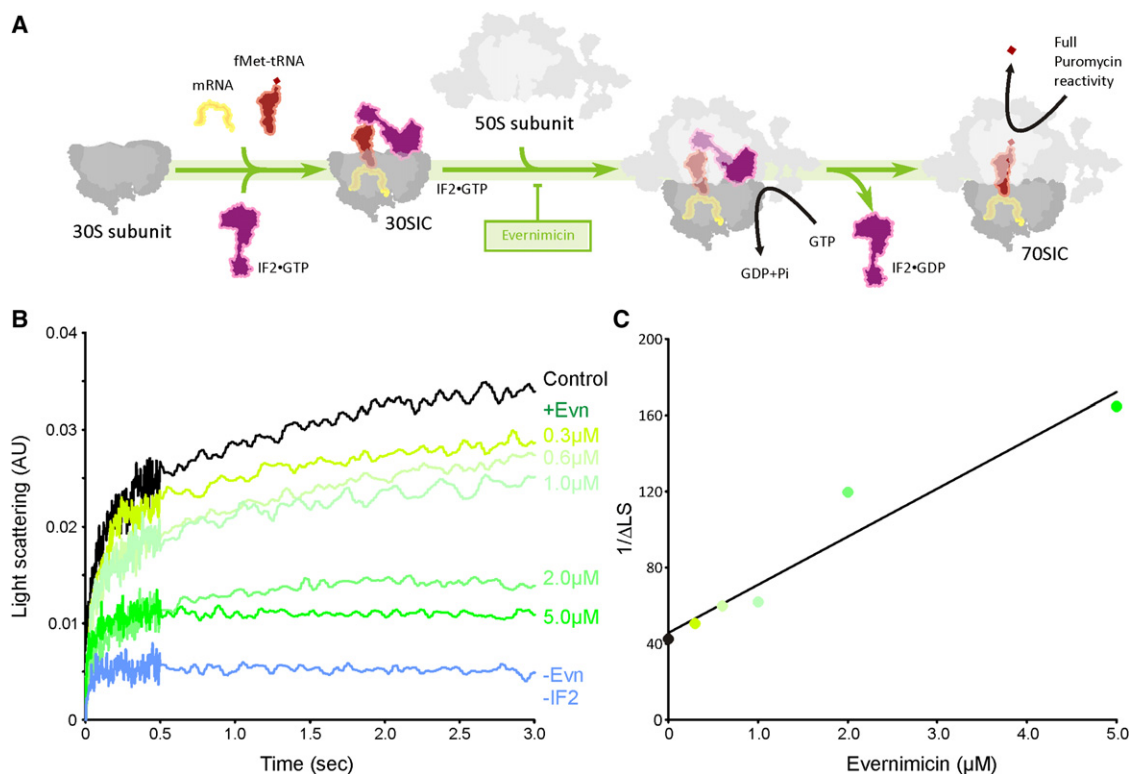
By contrast, translocation clearly proceeds via a two-step reaction for PRE complexes containing *E. coli* tRNA<sup>fMet</sup> in the P site and *E. coli* fMetPhe-tRNA<sup>Phe</sup> (Prf16/20) in the A site (Figure 3C) (Pan et al., 2007). In this case an initial rapid increase in fluorescence intensity is followed by a gradual decrease, with respective apparent rate constants of  $20.7 \pm 0.6$  s<sup>-1</sup> and  $1.3 \pm 0.1$  s<sup>-1</sup>. The presence of MiC abolishes almost all fluores-

cent change (Figure 3C), strongly inhibiting step 1 and, thus, step 2, as reported previously for ThS (Pan et al., 2007). In contrast, Evn does not inhibit the translocation reaction. In fact the apparent rate constants (step 1,  $28.1 \pm 0.7$  s<sup>-1</sup>; step 2,  $3.5 \pm 0.2$  s<sup>-1</sup>) suggest that the drug actually accelerates the process, particularly the second step (Figure 3C).

### Evn Inhibits IF2-Dependent 70SIC Formation

In bacteria, formation of the 70SIC involves the association of the large 50S subunit with a 30S-initiation complex (30SIC) comprising the 30S subunit, mRNA; initiator fMet-tRNA and three initiation factors IF1, IF2, and IF3 (Figure 4A) (reviewed by Laursen et al., 2005; Simonetti et al., 2009). Binding of the 50S subunit to the 30SIC stimulates the GTPase activity of IF2, leading to release of IF2•GDP and resulting in a puromycin-reactive 70SIC (Figure 4A) (Grigoriadou et al., 2007).

Evn has previously been shown to inhibit IF2-dependent formation of fMet-puromycin (Belova et al., 2001), leading to its classification as a translation initiation inhibitor. However, to our knowledge, the exact step of inhibition has not been determined. To examine this further, 70SIC formation has been monitored kinetically using light scattering as described previously



**Figure 4. Evn Inhibits IF2-Dependent 70SIC Formation**

(A) Scheme for 70SIC formation with site of Evn inhibition.

(B) 30SIC (0.3 μM) was premixed with various concentrations of Evn (0–5 μM) and then rapidly mixed with 50S subunits (0.3 μM) in a KinTek stopped-flow spectrophotometer. The sample from which IF2 was omitted demonstrates the dependence of 70SIC formation on IF2.

(C) A plot of the reciprocal of IF2-dependent light-scattering increase at 1 s versus Evn concentration, allowing calculation of an apparent  $K_i$  for Evn of  $1.8 \pm 0.2 \mu\text{M}$ .

(Grigoriadou et al., 2007): 30SIC programmed with 022AUG mRNA was rapidly mixed with 50S subunits, and the increase in light scattering due to 70SIC formation (black control trace in Figure 4B) was monitored using stopped-flow spectrophotometry. In the absence of IF2, no increase in light scattering was observed (blue trace in Figure 4B), illustrating the IF2-dependence for 70SIC reported previously (Antoun et al., 2006; Grigoriadou et al., 2007). Pre-incubation of the 30SIC with increasing concentrations of Evn before 50S addition led to a corresponding decrease in IF2-dependent light scattering (green traces in Figure 4B). At 5 μM Evn, almost all IF2-dependent light scattering was abolished, indicating that Evn inhibits the IF2-dependent association of the 30SIC with the 50S subunit. A plot of the reciprocal of the increase of IF2-dependent light scattering at 1 s versus Evn concentration (Figure 4C) yields an apparent  $K_i$  for Evn of  $1.8 \pm 0.2 \mu\text{M}$ .

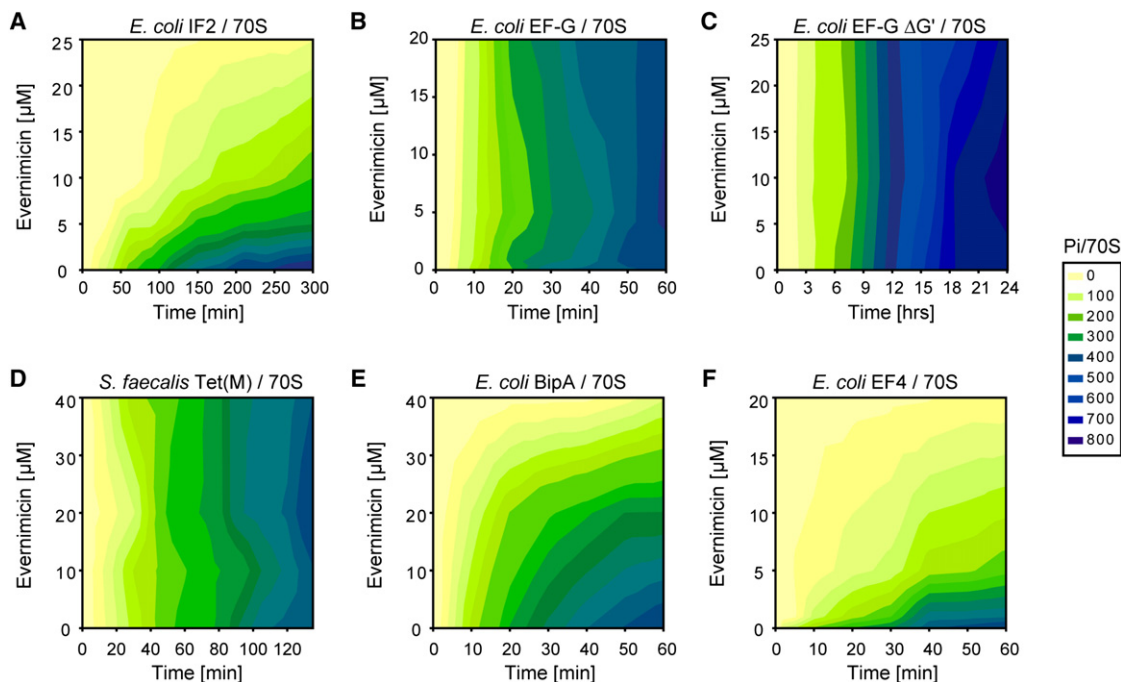
#### Differential Effects of Evn on GTPase Activity of Translational GTPases

Evn strongly inhibits the rdGTPase activity of IF2 (Figure 5A) but has little or no effect on the rdGTPase activities of EF-G (Figure 5B), or EF-GΔG' (Figure 5C), or of the EF-G paralog Tet(M) (Figure 5D), consistent with the potency of Evn in inhibiting 70SIC formation (Figure 4B) and with the lack of effect of Evn on EF-G-dependent translocation (Figure 3C). Surprisingly, Evn

was also found to inhibit the rdGTPase of BipA (Figure 5E) and especially EF4 (Figure 5F). Indeed, the inhibitory activity of Evn toward EF4 ( $\text{IC}_{50} = \sim 3 \mu\text{M}$ ) was higher than that toward IF2 ( $\text{IC}_{50} = \sim 7 \mu\text{M}$ ), and much higher than toward BipA ( $\text{IC}_{50} = \sim 20 \mu\text{M}$ ).

#### EF4-Dependent Back-Translocation Is Inhibited by Evn

EF4 catalyzes partial back-translocation, i.e., the movements of mRNA and tRNAs from POST toward the PRE state (Figure 6A) (Liu et al., 2010; Qin et al., 2006). As described previously (Liu et al., 2010), EF4-mediated partial back-translocation was monitored using prf-labeled tRNAs: POST state ribosomes containing fMetPhe-tRNA<sup>Phe</sup>(Prf16/20) in the P site and tRNA<sup>fMet</sup> in the E site were rapidly mixed in a stopped-flow spectrophotometer with EF4 and GDPNP, with fluorescence change being monitored over time (Figure 6B). In the absence of antibiotic, back-translocation proceeds via a three-step process (blue trace in Figure 6B) consistent with movement through a series of three intermediate states as reported (Liu et al., 2010) (INT1–3 in Figure 6A). In the presence of increasing concentrations of Evn, both the fluorescence change and rates of each step in the partial back-translocation were inhibited by Evn, (Figure 6B), leading to an apparent  $K_i$  for Evn binding to the POST complex of 0.6 μM (Figure 6C). High concentrations of Evn (5 μM in Figure 6B) completely abolished the EF4-catalyzed component of



**Figure 5. Effect of Evn on the GTPase Activity of Various Translation Factors**

(A–F) Activation of uncoupled rdGTPases of translation factors (A) *E. coli* IF2, (B) *E. coli* EF-G, (C) *E. coli* EF-GΔG', (D) *S. faecalis* TetM, (E) *E. coli* BipA, and (F) *E. coli* EF4, in the presence of increasing concentrations of Evn. The inset shows the color gradient scale from 0 (yellow) to 800 pmol (blue) of Pi produced per pmol of 70S ribosomes, following subtraction of background intrinsic GTPase activities.

the back-translocation, and only the two-step spontaneous reverse-translocation process that occurs in the absence of EF4 was observed (Liu et al., 2010). Evn inhibition of EF4-dependent partial back-translocation is similar to that reported earlier using spectinomycin (Liu et al., 2010), a well-characterized translocation inhibitor (Wilson, 2009), and is consistent with the observation that Evn is a potent inhibitor of the EF4 rdGTPase activity (Figure 5F).

## DISCUSSION

### Influence of Thiopeptides on Translocation and Translation Factor GTPase Activities

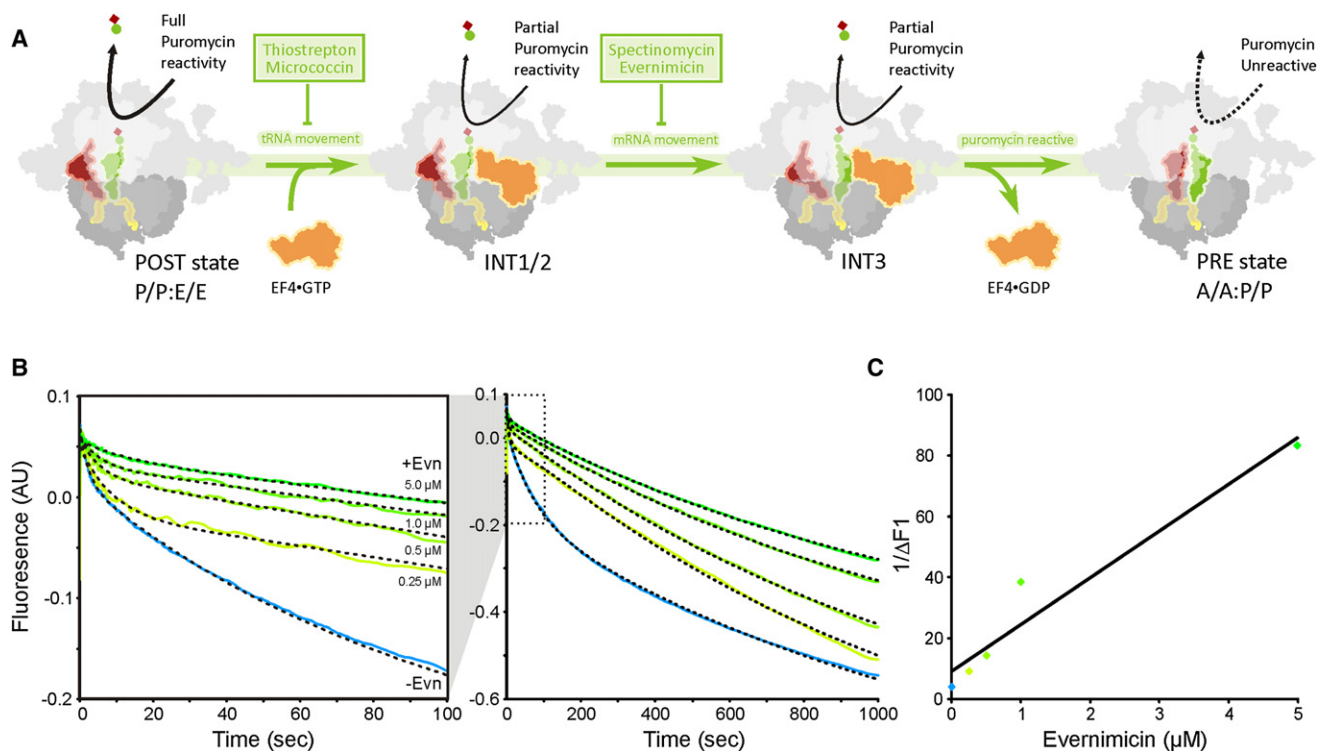
Although both ThS and MiC inhibit the multiple-turnover rdGTPase activities of IF2, TetM, EF4, and BipA (Figures 2B–2E), MiC differs from ThS in stimulating the rdGTPase of EF-G, an activity that is strongly inhibited by ThS (Figure 2A). Our results suggest that ThS inhibition and MiC stimulation of EF-G rdGTPase arise ultimately from differential effects on the interactions between the G' subdomain of EF-G and L7-CTD.

ThS allows ribosome-binding and single-turnover GTPase activity of EF-G but prevents the stable accommodation of EF-G on the ribosome, which is necessary for tRNA translocation (Rodnina et al., 1999; Seo et al., 2006). Part of the accommodation of EF-G encompasses the movement of EF-G toward L11-NTD, which is inhibited by ThS (Seo et al., 2006), consistent with the structural overlap between the binding site of ThS and domain V of EF-G, both locating to the cleft formed by H43/44 and L11-NTD (Figure 1F) (Harms et al., 2008). Because ThS

also prevents multiple-turnover GTPase activity of EF-G by inhibiting Pi release (Savelsbergh et al., 2003; Seo et al., 2006), EF-G remains trapped on the ribosome but in an unaccommodated state. In contrast, MiC allows Pi release from EF-G (Starosta et al., 2009) and, thus, stimulates the multiple-turnover rdGTPase activity of EF-G, as observed here (Figure 2A) and reported previously (Cameron et al., 2002; Cundliffe and Thompson, 1981; Lentzen et al., 2003). In agreement with the idea that MiC stimulates the rdGTPase of EF-G by stabilization of the interaction between the L7-CTD and the G' subdomain of EF-G (Harms et al., 2008), we could show that the MiC-dependent stimulation of EF-G rdGTPase activity was lost when the G' subdomain of EF-G was removed (Figure 2F) or the ribosomes lacked L7/L12 (Figures 2G–2I). Moreover, the rdGTPase activities of translation factors that naturally lack or have a reduced G' subdomain (TetM, EF4, BipA, IF2) were also inhibited by MiC (Figures 2B–2E).

Although many translation factors lack the complete G' subdomain, NMR studies indicate that IF2, EF-Tu, and RF3 interact with the same conserved region of L7-CTD as EF-G (Helgstrand et al., 2007). L7/L12 has been proposed to interact with helix αD<sub>1</sub> of domain I of EF-Tu. However, this interaction is more important for initial binding of EF-Tu·GTP·aa-tRNA to the ribosome, rather than for subsequent steps, such as A-site binding and GTPase activation (Kothe et al., 2004). Nevertheless, like EF-G, the rdGTPase activity of EF-Tu is dramatically reduced when ribosomes are depleted of L7/L12 (Diaconu et al., 2005; Mohr et al., 2002). In contrast, depletion of L7/L12 reduces the rate of association of IF2 with the ribosome, rather than directly





**Figure 6. Evn Inhibits EF4-Mediated Back-Translocation**

(A) Scheme for EF4-catalyzed back-translocation with sites of antibiotic inhibition.

(B) Fluorescence changes over different time scales. Isolated POST complex (0.1  $\mu\text{M}$ ) containing fMetPhe-tRNA<sup>Phe</sup>(Prf) in the P site and tRNA<sup>fMet</sup> in the E site, premixed with 0.15  $\mu\text{M}$  tRNA<sup>fMet</sup>, to increase E-site occupancy, were rapidly mixed in a stopped-flow spectrophotometer with 3  $\mu\text{M}$  EF4, 0.5 mM GDPNP, and different concentrations of Evn as indicated on the figure. All concentrations are final after mixing. Lines through the traces are fit to a kinetic model (Liu et al., 2010) in which back-translocation proceeds via a three-step process in the absence of Evn (the first two of which are catalyzed by EF4) and via a two-step process at saturating Evn.

(C) A plot of the reciprocal of the apparent magnitude of the fluorescence change for the second, EF4-catalyzed step versus Evn concentration, giving an apparent  $K_i$  for Evn binding to the POST complex of  $0.6 \pm 0.1 \mu\text{M}$ .

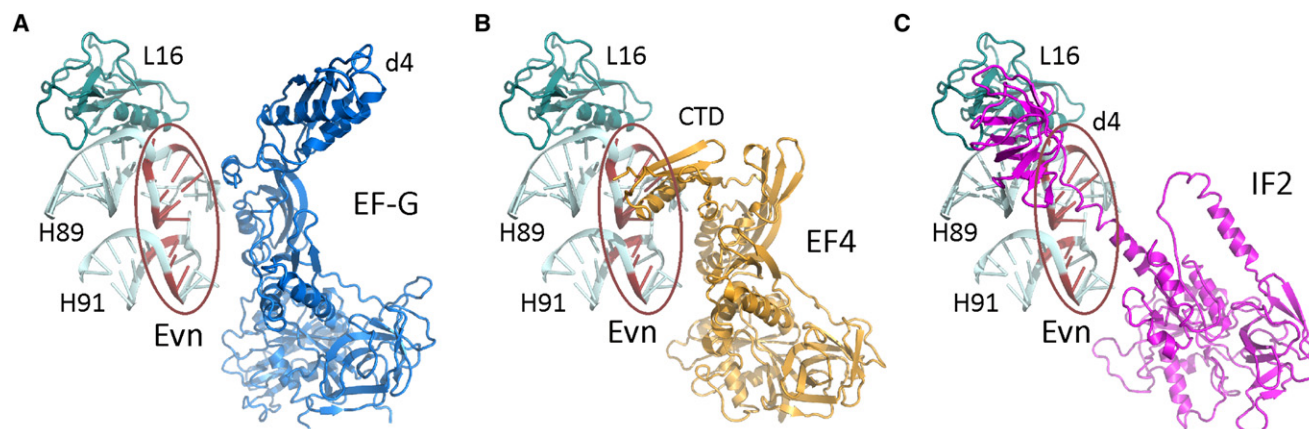
affecting GTP hydrolysis and Pi release (Huang et al., 2010). We observed that the rdGTPase of LepA and BipA with 70SΔL7/L12 was significantly reduced but was also inhibited by both MiC and ThS (Figures 2H and 2I). Further work will be needed to distinguish between factor binding versus GTPase activity defects.

Despite the contrasting effects of MiC and ThS on the GTPase activities of EF-G (Figure 2A) (Cameron et al., 2002; Cundliffe and Thompson, 1981; Lentzen et al., 2003), our kinetic analysis demonstrates that MiC, like ThS, is a potent inhibitor of the translocation reaction (Figure 3B). The finding that MiC targets translocation is in agreement with original conclusions of Pestka and Brot (1971), which were based upon its inhibition of poly(U)-dependent poly(Phe) synthesis but its lack of affect on either aa-tRNA binding or peptide-bond formation. Given the similarity in binding site between MiC and ThS, it thus appears likely that both MiC and ThS inhibit translocation analogously—namely, by preventing the transition of EF-G from an initially weaker binding state to a fully accommodated state on the ribosome, which, we would suggest, is necessary for translocation (Seo et al., 2006). We note that despite their diverse effects on the rdGTPase of EF-G, the inhibitory potency of MiC and ThS with respect to in vitro transcription-translation systems is comparable (both have an  $\text{IC}_{50}$  of  $\sim 3 \mu\text{M}$ ) (Figure S3).

### Influence of the Orthosomycin Evn on Translation Factor Activities

Unlike thiopeptides, we find that the orthosomycin Evn has no inhibitory effect on rdGTPase activity of EF-G (Figure 5B) or on the EF-G-mediated translocation reaction (Figure 3C). This is consistent with the lack of overlap between the putative Evn-binding site and the binding position of EF-G determined by structural studies (Connell et al., 2007; Gao et al., 2009; Ratje et al., 2010) (Figure 7A). Similarly, Evn does not inhibit the rdGTPase of Tet(M) (Figure 5D) or of Tet(O) (data not shown), which interacts with the ribosome in an analogous manner to EF-G (Spahn et al., 2001). In contrast we find that Evn is a potent inhibitor of the rdGTPase activity of IF2 (Figure 5A), BipA, and EF4 (Figures 5E and 5F). Although little is known about the structure or function of BipA on the ribosome (deLivron et al., 2009), structures of EF4 alone (Evans et al., 2008) and bound to the ribosome (Connell et al., 2008) reveal an overall similarity with EF-G. One exception is the unique CTD of EF4 (Evans et al., 2008), which on the ribosome is oriented back toward the large subunit (Connell et al., 2008) and encroaches upon the Evn-binding site (Figure 7B). Such overlap is consistent with our finding that Evn is a potent inhibitor EF4-mediated back-translocation reaction (Figure 6B). Our results demonstrating potent





**Figure 7. Putative Binding Sites of Orthosomycin Antibiotics Relative to Translation Factors EF-G, EF4, and IF2**

(A–C) Relative position of (A) EF-G (blue) (Connell et al., 2007; Gao et al., 2009), (B) EF4 (orange) (Connell et al., 2008), and (C) IF2 (purple) (Allen et al., 2005; Marzi et al., 2003) to the putative Evn-binding site (encircled in red) (Belova et al., 2001; Wilson, 2009). Nucleotides associated with Evn binding in H89 and H91 are colored red, and L16 (teal) is shown for reference. Note the overlap in positions of the CTD of EF4 and domain 4 (d4) of IF2 with the putative Evn-binding site.

Evn inhibitory effects on noninitiation translation factors, such as BipA and EF4, suggest that Evn can no longer be considered exclusively as an initiation inhibitor, as it has been heretofore. Indeed, Evn is a slightly stronger inhibitor of the rdGTPase activity of EF4 compared with IF2, and the  $K_i$  (0.6  $\mu$ M) for Evn binding to the POST complex (Figure 6C) is a little lower than the  $K_i$  (1.8  $\mu$ M) for Evn inhibition of IF2-dependent 70SIC formation.

Nevertheless, because the gene for EF4 is not essential for survival in *E. coli* (Dibb and Wolfe, 1986), the principal antimicrobial target of Evn is most likely IF2. Here, we demonstrate that Evn can inhibit the rdGTPase activity of IF2 (Figure 5A) as well as prevent the IF2-dependent association of the 30SIC with the large ribosomal subunit (Figure 4B). These findings support an earlier suggestion that Evn inhibits 70SIC formation, which was based on the ability of Evn to prevent the formation of fMet-puromycin in an IF2-dependent manner (Belova et al., 2001). Models for IF2 bound to the ribosome derived from biochemical (Marzi et al., 2003) and cryo-EM data (Allen et al., 2005; Myasnikov et al., 2005) suggest that domain 4 of IF2 and the associated linker region encroach on the Evn-binding site (Figure 7C). Thus, we believe that Evn sterically interferes with IF2 binding to the large ribosomal subunit, accounting for the Evn-dependent reduction in rdGTPase activity of IF2 with 70S ribosomes (Figure 5A) as well as the reduction in 70SIC as observed using light scattering (Figure 4B). The similarity between the  $K_i$  (1.8  $\mu$ M) of Evn inhibition for 70SIC formation and the half-inhibitory concentration ( $IC_{50} = \sim 2 \mu$ M) of Evn for synthesis of GFP as measured in an *E. coli* in vitro-coupled transcription-translation system (Figure S3) also supports the claim that Evn targets predominantly the initiation phase of protein synthesis (Belova et al., 2001).

## SIGNIFICANCE

**Insight into the mechanism of action of diverse classes of antibiotics, such as the thiopeptides and orthosomycins, to inhibit distinct steps during translation can provide**

**insight into the fundamental process of translation. Here, we demonstrate that although the thiopeptides MiC and ThS have contrasting effects on the rdGTPase activity of EF-G, both antibiotics are potent inhibitors of EF-G-dependent translocation reaction. Our results demonstrate that the MiC-dependent stimulation of the rdGTPase of EF-G requires the presence of the G' subdomain of EF-G as well as ribosomal proteins L7/L12. This finding supports the idea that recycling of EF-G from the ribosome, which occurs upon release of  $P_i$ , is mediated via the interaction of L7-CTD with the G' subdomain of EF-G. In contrast we can demonstrate that Evn does not influence EF-G rdGTPase, or EF-G dependent translocation, but is a potent inhibitor of EF4-dependent back-translocation reaction as well as IF2-dependent 70S-initiation complex formation. These findings are in agreement with the predicted binding site of the orthosomycins relative to the binding sites of EF-G, EF4, and IF2 on the ribosome. Understanding mechanistically how antibiotics perturb the translational apparatus is an important step for the future development of new improved antimicrobial agents to overcome the emerging resistant bacterial pathogens.**

## EXPERIMENTAL PROCEDURES

### Component Preparation

ThS was purchased from Sigma, MiC P1 was a kind gift of Dr. Torsten Stachelhaus, and gDNA from *S. faecalis* was kindly provided by Dr. Vincent Perrelet. *E. coli fusA* and *bipA*, *S. faecalis tetM* as well as *T. thermophilus* (HB8) *fusA* full-length genes were cloned into pET-46 Ek/LIC vector, and *E. coli infB* gene was cloned into pET-14b, in accordance with the manufacturer's instructions (Novagen). EF-GΔG' mutants were prepared using QuikChange Site-Directed Mutagenesis Kit (Stratagene). *E. coli* EF4 was expressed in pET14b as described previously (Qin et al., 2006). Recombinant proteins were expressed in BL21 (DE3) cells, at 20°C with 0.2 mM IPTG, then purified with a  $Ni^{2+}$ -NTA affinity column (QIAGEN), followed by gel-filtration chromatography on a HiLoad 16/60 Superdex 75 prep grade column (Amersham-Pharmacia) in a buffer containing 10 mM Tris (pH 7.8), 100 mM NaCl, and 10 mM 2-mercaptoethanol. *E. coli* 70S ribosomes lacking L7/L12 were prepared as described by Hamel et al. (1972) and Wystup et al. (1979). Tight-coupled *E. coli*

ribosomes, cloned *E. coli* His-tagged proteins EF-G, EF-Tu, IF1, IF2, and IF3, and *E. coli* [<sup>35</sup>S]fMet-tRNA<sup>fMet</sup>; *E. coli* [<sup>3</sup>H]Phe-tRNA<sup>Phe</sup> were prepared as described (Liu et al., 2010). MFK-mRNA was purchased from Dharmacon (Lafayette, CO, USA) with sequences 5'-GGG AAG GAG GUA AAA AUG UUU AAA CGU AAA UCU ACU-3' (initiator codon underlined).

#### IF2-Dependent 70SIC Formation Light-Scattering Assay

This assay was performed as described (Grigoriadou et al., 2007). 30SIC was formed by mixing 0.3 μM 30S, 0.45 μM IF1, 0.45 μM IF3, 0.45 μM fMet-tRNA<sup>fMet</sup>, 0.15 μM IF2, 0.9 μM AUG022-mRNA (Grigoriadou et al., 2007), and 100 μM GTP, premixed with various concentrations of Evn (0–5 μM) and then rapidly mixed with 50S subunits (0.3 μM) in a KinTek stopped-flow spectrophotometer. Excitation was at 436 nm, and light scattering was determined using a 455 nm cutoff filter. All concentrations are final after mixing.

#### Back-Translocation Assay

All of the following complexes were made up in buffer A (20 mM HEPES-KOH [pH 7.5], at 0°C, 150 mM NH<sub>4</sub>Ac, 4.5 mM MgAc<sub>2</sub>, 4 mM β-mercaptoethanol, 0.05 mM spermine, and 2 mM spermidine) at 37°C. Initiation complex was formed by incubating WT ribosomes (2 μM) with mRNA MFK (8 μM), IF1 (3 μM), IF2 (3 μM), IF3 (3 μM), GTP (1 mM), and [<sup>35</sup>S]fMet-tRNA<sup>fMet</sup> (3 μM) for 25 min. Ternary complex was formed by incubating EF-Tu (6 μM) with labeled Phe-tRNA<sup>Phe</sup> (3 μM), GTP (1 mM), phosphoenolpyruvate (Roche Diagnostics) (1.5 mM), and pyruvate kinase (Roche Diagnostics) (0.015 mg/ml) for 15 min. POST complexes were formed by incubating ternary complex and initiation complex at 37°C briefly for 45 s and then in the presence of EF-G (molar ratio of EF-G:ribosome was 0.2:1) and GTP (1 mM) at 37°C for 10 min. Then they were purified by ultracentrifugation through a 1.1 M Sucrose cushion in buffer A (450,000 × g, 40 min, 4°C). POST complex concentration was calculated from the amount of ribosome-bound fMet-[<sup>3</sup>H]Phe-tRNA<sup>Phe</sup>. Stopped-flow fluorescence experiments were performed using an SX.18MV Stopped-Flow Spectrofluorometer (Applied Photophysics). POST complex (0.1 μM) containing fMetPhe-tRNA<sup>Phe</sup>(Prf) in the P site and tRNA<sup>fMet</sup> in the E site was rapidly mixed with 0.15 μM tRNA<sup>fMet</sup>, 3 μM EF4•GDPNP, and various concentrations of Evn (0–5 μM). prf was excited at 460 nm, and fluorescence was monitored using a 495 nm long-pass filter. Lines through the data are fit to triple-exponential equations using the program IGOR Pro (WaveMetrics).

#### Translocation Assay

PRE complexes were formed by incubating initiation complex and ternary complex at 37°C for 45 s. Then they were purified by ultracentrifugation through a 1.1 M Sucrose cushion in buffer A with 20 mM Mg<sup>2+</sup> (450,000 × g, 40 min, 4°C). PRE complex concentration was calculated from the amount of ribosome-bound fMet-[<sup>3</sup>H]Phe-tRNA<sup>Phe</sup>. Stopped-flow fluorescence experiments were performed using an SX.18MV Stopped-Flow spectrofluorometer. prf was excited at 460 nm, and fluorescence was monitored using a 495 nm long-pass filter. Data are fit to double-exponential equations using the program IGOR Pro (WaveMetrics).

#### Malachite Green GTPase Activity Assays

GTPase activity was measured using the Malachite Green Phosphate Kit (BioAssay) that quantifies the green complex formed between malachite green, molybdate, and free orthophosphate. Unless otherwise mentioned, all reactions contained 30 nM *E. coli* 70S ribosomes, 20 μM GTP, and 60 nM protein in the presence or absence of antibiotics as necessary. Reactions were transferred into 96-well microtiter plates, and color formation was measured on Tecan Infinite M1000 microplate reader at 650 nm. Reactions performed in the absence of ribosomes were used as a background signal to account for the intrinsic GTPase activity of the translation factor.

#### In Vitro Transcription-Translation Assay

All coupled transcription-translation experiments were performed using an *E. coli* lysate-based system in the presence and absence of antibiotics as described previously (Starosta et al., 2009, 2010). Reactions were transferred into 96-well microtiter plates, and the GFP fluorescence was measured with a Typhoon Scanner 9400 (Amersham Bioscience) using a Typhoon blue laser module (Amersham Bioscience). Images were then quantified using Image-

Quant TL (GE Healthcare) and represented graphically using SigmaPlot (Systat Software, Inc.).

#### Figure Preparation

Chemical structures for the precursor compounds were drawn using ChemDraw (Advanced Chemistry Development, Inc., Toronto, Canada), and all structural figures were prepared with PyMOL (<http://www.pymol.org>).

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.chembiol.2011.03.010.

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#### REFERENCES

- Aarestrup, F.M., and Jensen, L.B. (2000). Presence of variations in ribosomal protein L16 corresponding to susceptibility of *enterococci* to oligosaccharides (Avilamycin and evernimicin). *Antimicrob. Agents Chemother.* 44, 3425–3427.
- Adrian, P.V., Mendrick, C., Loebenberg, D., McNicholas, P., Shaw, K.J., Klugman, K.P., Hare, R.S., and Black, T.A. (2000a). Evernimicin (SCH27899) inhibits a novel ribosome target site: analysis of 23S ribosomal DNA mutants. *Antimicrob. Agents Chemother.* 44, 3101–3106.
- Adrian, P.V., Zhao, W., Black, T.A., Shaw, K.J., Hare, R.S., and Klugman, K.P. (2000b). Mutations in ribosomal protein L16 conferring reduced susceptibility to evernimicin (SCH27899): implications for mechanism of action. *Antimicrob. Agents Chemother.* 44, 732–738.
- Allen, G.S., Zavialov, A., Gursky, R., Ehrenberg, M., and Frank, J. (2005). The cryo-EM structure of a translation initiation complex from *Escherichia coli*. *Cell* 121, 703–712.
- Antoun, A., Pavlov, M.Y., Lovmar, M., and Ehrenberg, M. (2006). How initiation factors tune the rate of initiation of protein synthesis in bacteria. *EMBO J.* 25, 2539–2550.
- Bagley, M.C., Dale, J.W., Merritt, E.A., and Xiong, X. (2005). Thiopeptide antibiotics. *Chem. Rev.* 105, 685–714.
- Belova, L., Tenson, T., Xiong, L.Q., McNicholas, P.M., and Mankin, A.S. (2001). A novel site of antibiotic action in the ribosome: interaction of evernimicin with the large ribosomal subunit. *Proc. Natl. Acad. Sci. USA* 98, 3726–3731.
- Blanchard, S.C., Kim, H.D., Gonzalez, R.L., Jr., Puglisi, J.D., and Chu, S. (2004). tRNA dynamics on the ribosome during translation. *Proc. Natl. Acad. Sci. USA* 101, 12893–12898.
- Blanchard, S.C., Cooperman, B.S., and Wilson, D.N. (2010). Probing translation with small-molecule inhibitors. *Chem. Biol.* 17, 633–645.
- Brandi, L., Marzi, S., Fabbretti, A., Fleischer, C., Hill, W., Lodmell, J., and Gualerzi, C. (2004). The translation initiation functions of IF2: targets for thio-strepton inhibition. *J. Mol. Biol.* 335, 881–894.
- Cameron, D.M., Thompson, J., March, P.E., and Dahlberg, A.E. (2002). Initiation factor IF2, thio-strepton and micrococcin prevent the binding of elongation factor G to the *Escherichia coli* ribosome. *J. Mol. Biol.* 319, 27–35.
- Connell, S.R., Tracz, D.M., Nierhaus, K.H., and Taylor, D.E. (2003a). Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob. Agents Chemother.* 47, 3675–3681.

- Connell, S.R., Trieber, C.A., Dinios, G.P., Einfeldt, E., Taylor, D.E., and Nierhaus, K.H. (2003b). Mechanism of Tet(O)-mediated tetracycline resistance. *EMBO J.* 22, 945–953.
- Connell, S.R., Takemoto, C., Wilson, D.N., Wang, H., Murayama, K., Terada, T., Shirouzu, M., Rost, M., Schuler, M., Giesebrecht, J., et al. (2007). Structural basis for interaction of the ribosome with the switch regions of GTP-bound elongation factors. *Mol. Cell* 25, 751–764.
- Connell, S.R., Topf, M., Qin, Y., Wilson, D.N., Mielke, T., Fucini, P., Nierhaus, K.H., and Spahn, C.M. (2008). A new tRNA intermediate revealed on the ribosome during EF4-mediated back-translocation. *Nat. Struct. Mol. Biol.* 15, 910–915.
- Cundliffe, E., and Thompson, J. (1981). Concerning the mode of action of micrococin upon bacterial protein synthesis. *Eur. J. Biochem.* 118, 47–52.
- Dantley, K.A., Dannelly, H.K., and Burdett, V. (1998). Binding interaction between Tet(M) and the ribosome: requirements for binding. *J. Bacteriol.* 180, 4089–4092.
- Datta, P.P., Sharma, M.R., Qi, L., Frank, J., and Agrawal, R.K. (2005). Interaction of the G' domain of elongation factor G and the C-terminal domain of ribosomal protein L7/L12 during translocation as revealed by cryo-EM. *Mol. Cell* 20, 723–731.
- deLliron, M.A., and Robinson, V.L. (2008). Salmonella enterica serovar Typhimurium BipA exhibits two distinct ribosome binding modes. *J. Bacteriol.* 190, 5944–5952.
- deLliron, M.A., Mankanji, H.S., Lane, M.C., and Robinson, V.L. (2009). A novel domain in translational GTPase BipA mediates interaction with the 70S ribosome and influences GTP hydrolysis. *Biochemistry* 48, 10533–10541.
- Diaconu, M., Kothe, U., Schlunzen, F., Fischer, N., Harms, J.M., Tonevitsky, A.G., Stark, H., Rodnina, M.V., and Wahl, M.C. (2005). Structural basis for the function of the ribosomal L7/L12 stalk in factor binding and GTPase activation. *Cell* 121, 991–1004.
- Dibb, N.J., and Wolfe, P.B. (1986). *lep* operon proximal gene is not required for growth or secretion by *Escherichia coli*. *J. Bacteriol.* 166, 83–87.
- Evans, R.N., Blaha, G., Bailey, S., and Steitz, T.A. (2008). The structure of LepA, the ribosomal back translocase. *Proc. Natl. Acad. Sci. USA* 105, 4673–4678.
- Gao, Y.G., Selmer, M., Dunham, C.M., Weixlbaumer, A., Kelley, A.C., and Ramakrishnan, V. (2009). The structure of the ribosome with elongation factor G trapped in the posttranslocational state. *Science* 326, 694–699.
- Gonzalez, R.L., Jr., Chu, S., and Puglisi, J.D. (2007). Thiostrepton inhibition of tRNA delivery to the ribosome. *RNA* 13, 2091–2097.
- Grigoriadou, C., Marzi, S., Kirillov, S., Gualerzi, C.O., and Cooperman, B.S. (2007). A quantitative kinetic scheme for 70 S translation initiation complex formation. *J. Mol. Biol.* 373, 562–572.
- Grunberg-Manago, M., Dondon, J., and Graffe, M. (1972). Inhibition by thiostrepton of the IF-2-dependent ribosomal GTPase. *FEBS Lett.* 22, 217–221.
- Hamel, E., Koka, M., and Nakamoto, T. (1972). Requirement of an *E. coli* 50S ribosomal protein component for effective interaction of the ribosome with T and G factors and with guanosine triphosphate. *J. Biol. Chem.* 247, 805–814.
- Harms, J.M., Wilson, D.N., Schlunzen, F., Connell, S.R., Stachelhaus, T., Zaborowska, Z., Spahn, C.M., and Fucini, P. (2008). Translational regulation via L11: molecular switches on the ribosome turned on and off by thiostrepton and micrococin. *Mol. Cell* 30, 26–38.
- Helgstrand, M., Mandava, C.S., Mulder, F.A., Liljas, A., Sanyal, S., and Akke, M. (2007). The ribosomal stalk binds to translation factors IF2, EF-Tu, EF-G and RF3 via a conserved region of the L12 C-terminal domain. *J. Mol. Biol.* 365, 468–479.
- Huang, C., Mandava, C.S., and Sanyal, S. (2010). The ribosomal stalk plays a key role in IF2-mediated association of the ribosomal subunits. *J. Mol. Biol.* 399, 145–153.
- Hughes, R.A., and Moody, C.J. (2007). From amino acids to heteroaromatics—thiopeptide antibiotics, nature's heterocyclic peptides. *Angew. Chem. Int. Ed. Engl.* 46, 7930–7954.
- Kischa, K., Möller, W., and Stöffler, G. (1971). Reconstitution of a GTPase activity by a 50S ribosomal protein from *E. coli*. *Nat. New Biol.* 233, 62–63.
- Knutsson Jenvert, R.M., and Holmberg Schiavone, L. (2005). Characterization of the tRNA and ribosome-dependent pppGpp-synthesis by recombinant stringent factor from *Escherichia coli*. *FEBS J.* 272, 685–695.
- Kofoed, C.B., and Vester, B. (2002). Interaction of avilamycin with ribosomes and resistance caused by mutations in 23S rRNA. *Antimicrob. Agents Chemother.* 46, 3339–3342.
- Kothe, U., Wieden, H.J., Mohr, D., and Rodnina, M.V. (2004). Interaction of elongation factor Tu with helices 4 and 5 of protein L7/L12 on the ribosome. *J. Mol. Biol.* 336, 1011–1021.
- Laurberg, M., Kristensen, O., Martemyanov, K., Gudkov, A.T., Nagaev, I., Hughes, D., and Liljas, A. (2000). Structure of a mutant EF-G reveals domain III and possibly the fusidic acid binding site. *J. Mol. Biol.* 303, 593–603.
- Laursen, B.S., Sorensen, H.P., Mortensen, K.K., and Sperling-Petersen, H.U. (2005). Initiation of protein synthesis in bacteria. *Microbiol. Mol. Biol. Rev.* 69, 101–123.
- Lefranc, D., and Ciufolini, M.A. (2009). Total synthesis and stereochemical assignment of micrococin P1. *Angew. Chem. Int. Ed. Engl.* 48, 4198–4201.
- Lentzen, G., Klinck, R., Matassova, N., Aboul-ela, F., and Murchie, A. (2003). Structural basis for contrasting activities of ribosome binding thiazole antibiotics. *Chem. Biol.* 10, 769–778.
- Liu, H., Pan, D., Pech, M., and Cooperman, B.S. (2010). Interrupted catalysis: the EF4 (LepA) effect on back-translocation. *J. Mol. Biol.* 396, 1043–1052.
- Mann, P.A., Xiong, L., Mankin, A.S., Chau, A.S., Mendrick, C.A., Najarian, D.J., Cramer, C.A., Loebenberg, D., Coates, E., Murgolo, N.J., et al. (2001). EmtA, a rRNA methyltransferase conferring high-level evernimicin resistance. *Mol. Microbiol.* 41, 1349–1356.
- Marzi, S., Knight, W., Brandi, L., Caserta, E., Soboleva, N., Hill, W.E., Gualerzi, C.O., and Lodmell, J.S. (2003). Ribosomal localization of translation initiation factor IF2. *RNA* 9, 958–969.
- McConkey, G.A., Rogers, M.J., and McCutchan, T.F. (1997). Inhibition of Plasmodium falciparum protein synthesis. Targeting the plastid-like organelle with thiostrepton. *J. Biol. Chem.* 272, 2046–2049.
- McNicholas, P.M., Najarian, D.J., Mann, P.A., Hesk, D., Hare, R.S., Shaw, K.J., and Black, T.A. (2000). Evernimicin binds exclusively to the 50S ribosomal subunit and inhibits translation in cell-free systems derived from both Gram-positive and Gram-negative bacteria. *Antimicrob. Agents Chemother.* 44, 1121–1126.
- McNicholas, P.M., Mann, P.A., Najarian, D.J., Miesel, L., Hare, R.S., and Black, T.A. (2001). Effects of mutations in ribosomal protein L16 on susceptibility and accumulation of evernimicin. *Antimicrob. Agents Chemother.* 45, 79–83.
- Moazed, D., and Noller, H.F. (1989). Intermediate states in the movement of transfer RNA in the ribosome. *Nature* 342, 142–148.
- Modolell, J., Cabrer, B., Parmeggiani, A., and Vazquez, D. (1971). Inhibition by siomycin and thiostrepton of both aminoacyl-tRNA and factor G binding to ribosomes. *Proc. Natl. Acad. Sci. USA* 68, 1796–1800.
- Mohr, D., Wintermeyer, W., and Rodnina, M.V. (2002). GTPase activation of elongation factors Tu and G on the ribosome. *Biochemistry* 41, 12520–12528.
- Munro, J.B., Wasserman, M.R., Altman, R.B., Wang, L., and Blanchard, S.C. (2010). Correlated conformational events in EF-G and the ribosome regulate translocation. *Nat. Struct. Mol. Biol.* 17, 1470–1477.
- Myasnikov, A.G., Marzi, S., Simonetti, A., Giuliodori, A.M., Gualerzi, C.O., Yusupova, G., Yusupov, M., and Klaholz, B.P. (2005). Conformational transition of initiation factor 2 from the GTP- to GDP-bound state visualized on the ribosome. *Nat. Struct. Mol. Biol.* 12, 1145–1149.
- Nechifor, R., Murataliev, M., and Wilson, K.S. (2007). Functional interactions between the G' subdomain of bacterial translation factor EF-G and ribosomal protein L7/L12. *J. Biol. Chem.* 282, 36998–37005.
- Nicolaou, K.C., Safina, B.S., Zak, M., Lee, S.H., Nevalainen, M., Bella, M., Estrada, A.A., Funke, C., Zécri, F.J., and Bulat, S. (2005a). Total synthesis of thiostrepton. Retrosynthetic analysis and construction of key building blocks. *J. Am. Chem. Soc.* 127, 1159–1175.
- Nicolaou, K.C., Zak, M., Safina, B.S., Estrada, A.A., Lee, S.H., and Nevalainen, M. (2005b). Total synthesis of thiostrepton. Assembly of key building blocks and completion of the synthesis. *J. Am. Chem. Soc.* 127, 11176–11183.

- Nicolaou, K.C., Chen, J.S., Edmonds, D.J., and Estrada, A.A. (2009). Recent advances in the chemistry and biology of naturally occurring antibiotics. *Angew. Chem. Int. Ed. Engl.* **48**, 660–719.
- Otake, T., and Kaji, A. (1974). Micrococin: acceptor-site-specific inhibitor of protein synthesis. *Eur. J. Biochem.* **50**, 101–106.
- Owens, R.M., Pritchard, G., Skipp, P., Hodey, M., Connell, S.R., Nierhaus, K.H., and O'Connor, C.D. (2004). A dedicated translation factor controls the synthesis of the global regulator Fis. *EMBO J.* **23**, 3375–3385.
- Pan, D., Kirillov, S.V., and Cooperman, B.S. (2007). Kinetically competent intermediates in the translocation step of protein synthesis. *Mol. Cell* **25**, 519–529.
- Pestka, S. (1970). Thiostrepton: a ribosomal inhibitor of translocation. *Biochem. Biophys. Res. Commun.* **40**, 667–674.
- Pestka, S., and Brot, N. (1971). Studies on the formation of transfer ribonucleic acid-ribosome complexes. IV. Effect of antibiotics on steps of bacterial protein synthesis: some new ribosomal inhibitors of translocation. *J. Biol. Chem.* **246**, 7715–7722.
- Qin, Y., Polacek, N., Vesper, O., Staub, E., Einfeldt, E., Wilson, D.N., and Nierhaus, K.H. (2006). The highly conserved LepA is a ribosomal elongation factor that back-translocates the ribosome. *Cell* **127**, 721–733.
- Ratje, A., Loerke, J., Mikolajka, A., Br  nner, M., Hildebrand, P.W., Starosta, A.L., D  nh  fer, A., Connell, S.R., Fucini, P., Mielke, T., et al. (2010). Head swivel on the ribosome facilitates translocation by means of intra-subunit tRNA hybrid sites. *Nature* **468**, 713–716.
- Rodnina, M.V., Savelsbergh, A., Katunin, V.I., and Wintermeyer, W. (1997). Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome. *Nature* **385**, 37–41.
- Rodnina, M.V., Savelsbergh, A., Matassova, N.B., Katunin, V.I., Semenov, Y.P., and Wintermeyer, W. (1999). Thiostrepton inhibits the turnover but not the GTPase of elongation factor G on the ribosome. *Proc. Natl. Acad. Sci. USA* **96**, 9586–9590.
- Savelsbergh, A., Mohr, D., Wilden, B., Wintermeyer, W., and Rodnina, M.V. (2000). Stimulation of the GTPase activity of translation elongation factor G by ribosomal protein L7/12. *J. Biol. Chem.* **275**, 890–894.
- Savelsbergh, A., Katunin, V.I., Mohr, D., Peske, F., Rodnina, M.V., and Wintermeyer, W. (2003). An elongation factor G-induced ribosome rearrangement precedes tRNA-mRNA translocation. *Mol. Cell* **11**, 1517–1523.
- Savelsbergh, A., Mohr, D., Kothe, U., Wintermeyer, W., and Rodnina, M.V. (2005). Control of phosphate release from elongation factor G by ribosomal protein L7/12. *EMBO J.* **24**, 4316–4323.
- Schmeing, T.M., and Ramakrishnan, V. (2009). What recent ribosome structures have revealed about the mechanism of translation. *Nature* **461**, 1234–1242.
- Seo, H.S., Abedin, S., Kamp, D., Wilson, D.N., Nierhaus, K.H., and Cooperman, B.S. (2006). EF-G-dependent GTPase on the ribosome. Conformational change and fusidic acid inhibition. *Biochemistry* **45**, 2504–2514.
- Simonetti, A., Marzi, S., Jenner, L., Myasnikov, A., Romby, P., Yusupova, G., Klaholz, B.P., and Yusupov, M. (2009). A structural view of translation initiation in bacteria. *Cell. Mol. Life Sci.* **66**, 423–436.
- Sohmen, D., Harms, J.M., Schl  nzen, F., and Wilson, D.N. (2009a). Enhanced SnapShot: antibiotic inhibition of protein synthesis II. *Cell* **139**, 212–212.e1.
- Sohmen, D., Harms, J.M., Schl  nzen, F., and Wilson, D.N. (2009b). SnapShot: antibiotic inhibition of protein synthesis I. *Cell* **138**, 1248.e1.
- Spahn, C.M., Blaha, G., Agrawal, R.K., Penczek, P., Grassucci, R.A., Trieber, C.A., Connell, S.R., Taylor, D.E., Nierhaus, K.H., and Frank, J. (2001). Localization of the ribosomal protection protein Tet(O) on the ribosome and the mechanism of tetracycline resistance. *Mol. Cell* **7**, 1037–1045.
- Starosta, A.L., Qin, H., Mikolajka, A., Leung, G.Y., Schwinghammer, K., Nicolaou, K.C., Chen, D.Y., Cooperman, B.S., and Wilson, D.N. (2009). Identification of distinct thiopeptide-antibiotic precursor lead compounds using translation machinery assays. *Chem. Biol.* **16**, 1087–1096.
- Starosta, A.L., Karpenko, V.V., Shishkina, A.V., Mikolajka, A., Sumbatyan, N.V., Schl  nzen, F., Korshunova, G.A., Bogdanov, A.A., and Wilson, D.N. (2010). Interplay between the ribosomal tunnel, nascent chain, and macrolides influences drug inhibition. *Chem. Biol.* **17**, 1–10.
- Weisblum, B., and Demohn, V. (1970). Inhibition by thiostrepton of the formation of a ribosome-bound guanine nucleotide complex. *FEBS Lett.* **11**, 149–152.
- Wilson, D.N. (2009). The A-Z of bacterial translation inhibitors. *Crit. Rev. Biochem. Mol. Biol.* **44**, 393–433.
- Wolf, H. (1973). Avilamycin, an inhibitor of the 30S ribosomal subunits function. *FEBS Lett.* **36**, 181–186.
- Wystup, G., Teraoka, H., Schulze, H., Hampl, H., and Nierhaus, K.H. (1979). 50S subunit from *Escherichia coli* ribosomes. Isolation of active ribosomal proteins and protein complexes. *Eur. J. Biochem.* **100**, 101–113.
- Zarazaga, M., Tenorio, C., Del Campo, R., Ruiz-Larrea, F., and Torres, C. (2002). Mutations in ribosomal protein L16 and in 23S rRNA in *Enterococcus* strains for which evernimicin MICs differ. *Antimicrob. Agents Chemother.* **46**, 3657–3659.