Mechanism of elongation factor-G mediated fusidic acid resistance and fitness compensation in *Staphylococcus aureus*

Ravi Kiran Koripella, Yang Chen, Kristin Peisker, Cha San Koh, Maria Selmer* and Suparna Sanyal*

Department of Cell and Molecular Biology, Uppsala University, Box-596, BMC, 75124, Uppsala, Sweden

* Correspondence
Telephone: +46 18 4714220
Fax: +46 18 4714262
E-mail: suparna.sanyal@icm.uu.se and maria.selmer@icm.uu.se

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**Keywords:** fusidic acid, *Staphylococcus aureus*, EF-G, antibiotic resistance, fitness compensation

**Background:** Fitness loss and compensation in fusidic acid resistant EF-G mutants of *Staphylococcus aureus*.

**Results:** Slower translocation and ribosome-recycling due to restricted conformational change, plus increased tRNA drop-off cause fitness loss in F88L; these are recovered in F88L+M16I leading to fitness compensation.

**Conclusion:** Conformational dynamics of EF-G is crucial for its function.

**Significance:** Provides insight into how antibiotic resistant mutations affect in vivo fitness.

**SUMMARY:** Antibiotic resistance in bacteria is often associated with fitness loss, which is compensated by secondary mutations. Fusidic acid (FA), an antibiotic used against pathogenic bacteria *Staphylococcus aureus*, locks elongation factor-G (EF-G) to the ribosome after GTP hydrolysis. To clarify the mechanism of fitness loss and compensation in relation to FA resistance, we have characterized three *S. aureus* EF-G mutants with fast kinetics and crystal structures. Our results show that a significantly slower tRNA translocation and ribosome recycling, plus increased peptidyl-tRNA drop-off are the causes for fitness defect of the primary FA resistant mutant F88L. The double mutant F88L+M16I is three to four times faster than F88L in both reactions and showed no tRNA drop-off, explaining its fitness-compensatory phenotype. The M16I mutation alone showed hypersensitivity to FA, higher activity and somewhat increased affinity to GTP. The crystal structures demonstrate that F88 in switch II is a key residue for FA locking and also for triggering inter-domain movements in EF-G essential for its function, explaining functional deficiencies in F88L. The mutation M16I loosens the hydrophobic core in the G domain and affects domain I to domain II contact, resulting in improved activity both in the wild-type and F88L background. Thus, FA resistant EF-G mutations causing fitness loss and compensation operate by affecting the conformational dynamics of EF-G on the ribosome.

Since the discovery of penicillin, antibiotics have been the major weapon against microbial infections. With the wide use of antibiotics came the problem of antibiotic resistance, which led on one hand, to a continuous search for new antibiotics and on the other hand, to studies aiming at understanding the mechanism of antibiotic resistance. A common resistance mechanism involves point mutation in the drug target, which normally imposes some fitness cost in bacteria. However, the fitness costs are often overcome by creating additional mutations, commonly called...
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fitness-compensatory mutations (1-4). Thus, understanding the mechanism of fitness loss and compensation in association with antibiotic resistance is highly important to combat bacterial infections.

Fusidic acid (FA) is a narrow spectrum antibiotic used against the pathogenic Gram-positive bacterium Staphylococcus aureus since the 1960s. These bacteria, with humans as the favored host, have developed resistance against a wide spectrum of antibiotics, thereby leading to multidrug-resistant S. aureus (MRSA) strains, which showed comparatively lower rate of resistance gain against FA than other antibiotics (5). Thus FA is still used as an effective drug against Staphylococcal infections.

Fusidic acid works by blocking elongation factor – G (EF-G) on the ribosome with GDP. Since EF-G is crucial in two steps of translation, namely elongation and ribosome recycling, FA acts effectively by blocking either or both of these steps of bacterial protein synthesis (2,6-8). During elongation, FA traps EF-G on the ribosome following tRNA translocation and GTP hydrolysis. Similarly, during recycling, where multiple rounds of ribosome recycling factor (RRF) and EF-G action coupled with GTP hydrolysis are needed for splitting the 70S ribosome into the subunits (9,10), FA blocks EF-G turnover by fixing it on the ribosome with GDP. Based on in vitro fast kinetics it has been proposed that FA inhibition in ribosome recycling is more effective than in the elongation step (7,11). In vivo evidence supporting such claim is still lacking.

EF-G is a five-domain G protein. It undergoes large conformational changes upon binding to the 70S ribosome (12-15). The flexible switch I and switch II regions in the G domain of EF-G are primarily responsible for bringing about these changes through contacts with other domains, which ultimately drive the EF-G catalyzed translocation as well as ribosome recycling (13,16-18). The switch II region contains a set of highly conserved residues, including a Phe (F) at its tip, which is F88 in S. aureus and F90 in Thermus thermophilus. This residue is thought to be important for transmitting the conformational changes of switch II between the GTP and GDP forms of EF-G (19), although direct evidence has been lacking so far. As observed in the crystal structure of T. thermophilus EF-G locked to the 70S ribosome with FA, the drug interacts directly with F90 and prevents the switch II region of EF-G from going to its GDP conformation (13). On the other hand, FA can only bind when switch I has left its ordered GTP conformation. Thus, FA traps EF-G in an intermediate conformation between the GTP and GDP states and prevents its release from the post-translocation complex (13).

In S. aureus, a major set of mutations causing FA resistance are located in the fusa gene, encoding EF-G. Many of the FA resistant mutations are found scattered at the domain interfaces of EF-G (2,4,20), leading to the suggestion that FA binds to an interdomain pocket involving the tip of switch II (20). Indeed, FA could be seen in the hydrophobic pocket between domains I, II and III in the crystal structure of EF-G bound to the ribosome in the post-translocation state (13). One of the primary mutations that lead to strong FA resistance in clinical isolates of S. aureus is F88L (4). This mutation corresponds to the F90L mutation in T. thermophilus, which also confers FA resistance (21). The F88L mutant strain exhibited a significant growth defect (4) suggesting that F88 plays a crucial role in EF-G function. However, an additional mutation of the M16 residue (M16I or M16V) could compensate the fitness loss yet retaining FA resistance (4), through an unknown molecular mechanism.

In this study, we have characterized with steady-state and kinetic assays, wild type (WT) and the three mutant EF-Gs (F88L, F88L+M16I and M16I) from S. aureus, in various translation coupled and uncoupled reactions. In parallel, we have analyzed the crystal structures of all three mutants for understanding the structural basis for FA resistance and fitness compensation. To our knowledge, this is the first attempt to unravel the mechanism of fitness compensation associated with antibiotic resistance combining structural and functional tools. Our study shows how the crosstalk between different residues in EF-G influences its function and provides insights into the molecular mechanism of FA resistance, fitness loss and compensation in S. aureus.
EXPERIMENTAL PROCEDURES:

Plasmids and proteins:
The *fusA* gene was amplified from the total DNA of *S. aureus* strains WT (FDA486), or with mutations F88L and F88L+M16I, (provided by Prof. Diarmaid Hughes, Uppsala University) (4) and cloned in the vector pET30-Ek/Lic (Novagen). Further, the single mutation M16I was created by back mutating 88L to F in the double mutation construct F88L+M16I using the Quik-Change mutagenesis kit (Qiagen). Over-expression and purification of EF-G were carried out using the methodology of Chen et al. (22).

Components for biochemical experiments:
All translation components other than EF-G were from *E. coli*. MRE600 ribosomes, His-tagged translation factors, XR7 fMet-Phe-Phe-stop (MFF) mRNA and fMet-tRNA\textsubscript{Met} were purified as shown in Mandava et al. and Huang et al. (23,24). The experiments were performed in HEPES polymix buffer (pH 7.5) (24) at 37°C in the presence of energy pump components ATP (1 mM), GTP (1 mM), phosphoenolpyruvate (PEP) (10 mM), pyruvate kinase (PK) (50 µg/ml) and myokinase (MK) (2 µg/ml), resulting in a final Mg concentration of ~1.5 mM. FA was obtained from Leo Pharma (Denmark). tRNA\textsubscript{Phe} was purchased from Chemical Block (Moscow, Russia). Phosphoenolpyruvate (PEP), Pyruvatekinase (PK), Myokinase (MK) and nonradioactive amino acids were from Sigma-Aldrich. Radioactive amino acids and nucleotide triphosphates were from GE Healthcare.

*70S-EF-G-GDP-FA complex formation:*  
1 µM 70S ribosome, 5 µM EF-G, 10 µM [\textsuperscript{3}H]GTP and 0.5 mM fusidic acid were mixed in TAM buffer (10 mM Tris (pH 7.5), 10 mM ammonium chloride, 10 mM magnesium acetate and 0.3 mM fusidic acid) and incubated at 37°C for 5 minutes for complex formation. 50 µl of the mix was filtered through a nitrocellulose membrane filter (0.45 µM, Protran BA 85) presoaked in TAM, followed by washing with 5 ml of TAM buffer. The radioactive counts from the trapped [\textsuperscript{3}H]GDP remaining in the filter, indicated the amount of the 70S-EF-G-[\textsuperscript{3}H]GDP-FA complex formed when measured in a Beckman LS6500 scintillation counter.

*Binding and exchange of guanine nucleotides:*  
Association experiments - Mix A containing 2 µM of EF-G (WT and mutants) was rapidly mixed with equal volume of Mix B containing increasing concentration of N-methylanthraniloyl or mant-GDP (5 - 40 µM) or mant-GTP (20 - 100 µM). Exciting the fluorescence of mant group by fluorescence resonance energy transfer (FRET) between mant-GDP / GTP and the tryptophan residues located in the G domain of EF-G results in an increase in the fluorescence signal at 445 nm (Excitation at 280 nm), which was monitored against time in a stopped-flow apparatus (SX-20 Applied Photophysics) after passing through a KV390 cut-off filter (Schott). The apparent rates of binding (k\textsubscript{app}) were estimated by fitting the curves with single exponential using Origin 8.0 program. Linear fitting of k\textsubscript{app} values against the concentration of mant-GDP / GTP resolved into a straight line. Association and dissociation rate constants, k\textsubscript{on} and k\textsubscript{off} were determined from the slope of the plot and the Y axis intercept, respectively. The equilibrium dissociation constant (K\textsubscript{D}) was estimated from k\textsubscript{off} / k\textsubscript{on}.

Dissociation experiments – The k\textsubscript{off} was also estimated directly by chasing 4 µM mant-GDP / GTP in complex with 2 µM EF-G with 400 µM unlabeled nucleotides in a stopped-flow apparatus. The curves were fitted with single exponential using Origin 8.0.

*GTP hydrolysis and Pi release:*  
Multiple turnover ribosome stimulated GTP hydrolysis by EF-G(s) was initiated by mixing 1 µM EF-G to 4 µM 70S and 10 µM [\textsuperscript{3}H]-GTP. The reactions, quenched at different time-points with 50% formic acid, were analyzed in thin-layer chromatography (TLC). The extent of GTP hydrolyzed at each time-point was measured from the ratio of [\textsuperscript{3}H]-GDP to total count of [\textsuperscript{3}H]-GTP plus [\textsuperscript{3}H]-GDP and the rates were determined by fitting the data using single exponential function. GTP hydrolysis under single turnover condition was measured from Pi release, monitored by the increase in fluorescence of MDCC-labeled phosphate binding protein (PBP) (gift from Prof.
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Martin Webb, MRC, London, UK) in a stopped-flow instrument as described in (23,24). Mix A containing 0.5 µM ribosomes and 1 mM GTP was mixed with Mix B containing 2 µM EF-G and 2.5 µM MDCC-PBP at 37ºC and the fluorescence change with time was recorded using a 475 nm long pass filter. The rate of Pi release was obtained by fitting the initial burst phase with a single exponential.

**Tripeptide formation:**
70S initiation complex (IM) was formed by incubating 70S ribosomes (1 µM), [³H]fMet-tRNA⁹Met (1 µM), XR7 mRNA fMet-Phe-Phe-stop (MFF) (4 µM), initiation factors: IF1, IF2, IF3 (1 µM each) at 37ºC for 15 minutes. An elongation mix (EM) containing EF-Tu (10 µM), EF-Ts (5 µM), phenylalanine (200 µM), tRNA⁹Phe (5 µM), Phe-tRNA synthetase (0.2 units/µl), and EF-G (5 µM) was also incubated under similar conditions. Equal volumes of IM and EM were rapidly mixed in quench-flow (RQF-3 KinTek Corp) and the reaction was quenched at different time intervals by adding formic acid (17% final concentration). The pellet containing ribosome and all sorts of tRNAs (uncharged or carrying amino acid / peptide) was dissolved in 0.5M KOH to release the amino acid / peptides. The amount of tripeptide was analyzed in a RP-HPLC by comparing the [³H]tripeptide peak with the [³H]fMet peak and plotted against time. The highest yield (F88L + M16I) was normalized to 100%. All curves were fitted with single-exponential using ORIGIN 8.0.

**Peptidyl tRNA hydrolase (PTH) assay:**
PTH is an enzyme specific for ester bond hydrolysis of the peptidyl tRNAs off the ribosome. 100 µM PTH was added to the tripeptide reaction described above. After three minutes, the reaction was quenched with formic acid (17%). The supernatant containing peptides released from the dropped-off tRNAs and the pellet containing ribosomes with peptidyl / aminoacyl tRNAs bound were separately analyzed in a scintillation counter. The percentage of tRNA drop-off was estimated by comparing the [³H] count in the supernatant with the total count.

**Ribosome recycling:**
A termination complex (TC) containing 70S ribosomes (0.5 µM), XR7 mRNA: fMet-Phe-Leu-stop (2 µM), tRNA⁹Phe (3 µM) was rapidly mixed in a stopped flow apparatus to a mix containing RRF (10 µM), EF-G (5 µM) and IF3 (2 µM) in HEPES-polymix containing EPC at 37ºC. The kinetics of splitting of 70S ribosome into subunits was monitored by measuring the Rayleigh light scattering as described in Pavlov et al. (9).

**Luciferase synthesis in a Reconstituted Transcription-Translation-Folding (RTTF) system:**
The RTTF system is composed of purified translation components (ribosome, 20 aminoacyl-tRNA synthetases and translation factors) from *E. coli* along with T7 RNA polymerase, amino acids (Sigma) and energy pump components (24). In this work, *S. aureus* EF-G variants (7.5 µM) were used instead of *E. coli* EF-G. Synthesis of firefly luciferase was initiated by addition of pET30-Luc DNA and was followed by increase in luminescence measured in a Glo-Max 20/20 luminometer for 60 minutes at 37ºC without or with FA (250 µM).

**Crystallographic structure determination:**
Crystals of *S. aureus* EF-G F88L, M16I and F88L+M16I mutants were streak seeded from WT crystals into sitting drop vapor diffusion experiments under identical conditions as in (22). All data sets were collected at beamline ID23-1, ESRF (Grenoble, France), with an ADSC Quantum Q315r detector at 100K and 0.954 Å. Data were processed and scaled with the XDS package (25). The crystals belong to space group P2₁, X-ray data statistics are summarized in Table S1. The structures were solved by molecular replacement with PHASER (26), using domain I and II from *S. aureus* WT EF-G (pdb code 2XEX) as a starting model. Further model building was done in Coot (27) and refinements were performed with the CCP4 package (28) and Phenix (29). The quality of the structures was assessed using MolProbity (30). Refinement statistics are presented in Table S1. Sequence alignment was performed using ClustalW (31). Structure superposition was performed with O (32). All structure figures were generated using PyMOL.
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PDB codes:
The refined structures were deposited in the Protein Data Bank in Europe (PDBe) with the PDB codes of 3ZZ0 (M16I), 3ZZT (F88L) and 3ZZU (F88L/M16I).

RESULTS

Comparison of S. aureus EF-Gs in ribosomal complex formation with FA:
In the presence of 70S ribosomes, EF-G hydrolyses GTP and EF-G·GDP can be trapped on the 70S ribosome with 0.5 mM FA resulting in a 70S-EF-G·GDP-FA complex. Using [3H]GTP, this complex was detected quantitatively on the nitrocellulose filter. Highest complex formation was observed with the M16I mutant (normalized to 100%), similar to the WT (~90%). In comparison, only 5 - 8% complex was formed with F88L and F88L+M16I mutants, comparable with the background without 70S/EF-G/FA (>10%) (Figure 1A). This result, in line with in vivo characterization (4), shows that the F88L EF-Gs are modified in such a way that they no longer can be trapped with FA and lays the basis for their FA resistance.

Comparison of the EF-Gs in RTTF system:
We have compared S. aureus EF-G variants in an E. coli component based RTTF system (24) in the absence and presence of FA. Highest synthesis of active luciferase was obtained with the M16I mutant (normalized to 100%), while WT EF-G showed a moderate level (~60%) of synthesis (Figure 1B). In contrast, the FA resistant primary mutant F88L failed to produce any detectable luciferase indicating that it is highly defective in translation. We could not discern whether this was due to a defect in translocation or ribosome recycling or a combination of both. However, the double mutant F88L+M16I showed measurable luciferase synthesis (~10%) agreeing with its improved functionality in translation.

In the presence of FA (250 µM), the WT and M16I EF-Gs were completely inhibited and showed no luciferase synthesis (Figure 1B). Significantly, the F88L+M16I EF-G produced nearly the same level of luciferase as without FA, demonstrating the FA resistance associated with the F88L mutation.

Activity of the EF-G mutants in tRNA translocation:
One of the two major roles of EF-G is tRNA translocation during peptide elongation. We have compared the EF-Gs in single turnover tMet-Phe-Phe tripeptide (MFF) formation assay where an initiation complex containing 70S ribosome programmed with MFF coding mRNA and tMet-tRNA^{Met} was rapidly mixed in quench-flow with an elongation mix containing EF-Tu, Phe-tRNA^{Phe} and EF-G at 37°C. Under our experimental conditions, the formation of MF dipeptide took about 6 ms (k_{tripep} = 160 s^{-1}) (Figure S1A), and was not influenced by the presence of any of the EF-Gs. Assuming that formation of the second peptide bond will be equally fast as the first, any difference observed in the average time for tripeptide formation would arise essentially from the EF-G driven steps including tRNA translocation.

As shown in figure 2A, the rate of tripeptide formation using WT S. aureus EF-G was 0.67 ± 0.023 s^{-1}. The reaction was faster (k_{tripep} = 0.84 ± 0.028 s^{-1}) with the M16I mutant. Compared to these two, the FA-resistant mutant F88L was significantly slower (k_{tripep} = 0.07 ± 0.004 s^{-1}). However, the double mutant F88L+M16I was three times faster than F88L (k_{tripep} = 0.21 ± 0.015 s^{-1}), which explained its higher activity in RTTF as well as improved fitness in vivo (Table 1).

In the presence of 250 µM FA, strong inhibition of tripeptide formation was seen with the WT and the M16I EF-Gs (Figure 2B). As summarized in Table 1, the rate of tripeptide formation decreased ~18 times for the WT and ~40 times for the M16I mutant, but remained essentially unaltered for the F88L and the F88L+M16I mutants (Table 1), demonstrating FA resistance. The M16I mutation displayed the highest decrease, indicating hypersensitivity of this mutant towards FA.

We have further varied the concentration of FA in the tripeptide assay at a fixed time-point to estimate the half-maximal inhibitory concentration or IC_{50} for the EF-Gs (Figure 2C). While IC_{50} for the WT was 100 µM, it was lower for M16I (~50

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μM) indicating its higher sensitivity to FA. As expected, F88L showed higher IC₅₀ ~300 μM. Surprisingly, F88L+M16I showed a markedly higher IC₅₀ of ~1000 μM. This was somewhat unexpected since the in vivo estimate of its MIC (~150 μM) was comparable with that of F88L (~200 μM) (4). We noticed that a similar fitness compensatory mutant F88L+M16V also showed MIC in that range (250 μM) (4). When tested in our tripeptide assay, the F88L+M16I mutant behaved very much like the F88L+M16I mutant and produced IC₅₀ ~1000 μM (Figure S1B).

Peptidyl tRNA droff-off:
One notable feature of the F88L mutant was the lower yield of tripeptide (60%) compared to the other EF-Gs irrespective of the absence or presence of FA (Figure 2A, B). To check whether this low yield was due to drop-off of the dipeptidyl tRNA (33), the tripeptide assay was repeated in the presence of PTH, which cleaves off peptides only from free peptidyl-tRNAs outside the ribosome. As expected, about ~45% of the tRNAs dropped off with F88L EF-G when WT, M16I and F88L+M16I EF-Gs showed quite low (~15%) tRNA drop-off. A higher drop-off (~57%) was seen only when EF-G was absent suggesting that the dropped-off tRNAs are mostly dipeptidyl tRNAs from the A-site. It is known that the peptidyl tRNAs are comparatively unstable in the A-site and can drop-off more readily than from the P-site (21). Thus, it also indicates that the F88L mutation is majorly defective in the tRNA translocation step. The extent of tRNA drop-off remained same for all reactions independent of the presence of FA (Figure 2D) suggesting that FA plays no role in tRNA drop-off.

It is interesting to note that although tripeptide formation with the WT and the M16I EF-G became significantly slower in the presence of FA, there was no increase in tRNA drop-off (Figure 2B, Table 1). This suggests that even with FA these EF-Gs translocated tRNAs fast enough and could avoid tRNA drop-off. Thus, the rate limiting step in the tripeptide experiment for the WT and M16I in the presence of FA is EF-G release rather than translocation, which is otherwise rate limiting. It also confirms that FA does not block tRNA translocation, but inhibits release of EF-G from the ribosome (34,35).

Activity of EF-Gs in ribosome recycling:
EF-G has a second function in dissociation of the post-termination ribosomes into subunits, so-called ‘ribosome recycling’ together with ribosome recycling factor (RRF) (36-38). The subunit dissociation with different EF-Gs was monitored by Rayleigh light scattering in a stopped flow instrument (Figure 3). Consistent with their performance in the tripeptide assay, the F88L mutant produced the slowest rate of subunit dissociation (krecycle = 0.008 ± 0.001 s⁻¹) showing that this mutant is defective in the ribosome recycling step as well. In comparison, the fitness compensatory mutant F88L+M16I was about three fold faster (krecycle = 0.021 ± 0.001 s⁻¹) in recycling ribosomes. The WT and M16I mutant were 20 and 22 times faster than F88L and dissociated the ribosomes at a rate of 0.16 ± 0.012 s⁻¹ and 0.18 ± 0.007 s⁻¹, respectively. Since FA forms micelles at higher concentration which also scatter light, the effect of FA on subunit splitting could not be studied.

Affinity for guanine nucleotides (GDP/GTP):
The affinity of S. aureus WT and mutant EF-Gs towards GDP and GTP was estimated using mant-GDP/-GTP (39). Figures 4A and B illustrate the association experiments with S. aureus EF-Gs where increase in mant fluorescence upon mant-GDP/-GTP binding was monitored in stopped-flow. The apparent rates (kapp) derived from the binding experiments showed linear concentration dependence for mant-GDP (Figure 4C) and mant-GTP (Figure 4D). The association rate constants (kon) and the equilibrium dissociation constants (KD) for mant-GDP and –GTP are summarized in Table 2. All EF-G variants were highly similar in GDP binding with kon ~ 6 μM⁻¹ s⁻¹ and KD-mantGDP ~ 25 μM (Figure 4C). In contrast, some variation in KD-mantGTP was seen for the EF-G variants in GTP binding (Figure 4D). The FA resistant, fitness compromised mutant F88L showed lowest affinity for GTP (90 μM), whereas the FA hypersensitive, high activity mutant M16I showed highest affinity (63 μM). The WT and the F88L+M16I mutants showed intermediate values (Table 2).
The \( k_{\text{off-mantGDP}} \) and \( k_{\text{off-mantGTP}} \) were also estimated independently from the chase experiments (Figures 4E, F). In all cases, these values matched the ones determined from the Y intercept of the plots in Figures 4C and D. While \( k_{\text{off-mantGDP}} \) was quite similar, the \( k_{\text{off-mantGTP}} \) varied for different EF-Gs (Table 2). Interestingly, the dissociation of mant-GTP was slowest for M16I (97 ± 3.2 \( \text{s}^{-1} \)) (Figures 4D, F), contributing to its low \( K_{D-\text{mantGTP}} \). Putting these results together, the affinity to GTP (but not to GDP) seems to have a correlation with the activity of the EF-Gs.

**Activity in ribosome stimulated GTP hydrolysis:**

GTP hydrolysis was studied in single turnover condition by following Pi release in a stopped flow and in multiple turnover condition using TLC (see Experimental procedures for details). In both cases, the WT and mutant EF-Gs were highly similar to each other (Figures 5A, B); the rate of Pi release being ~45 \( \text{s}^{-1} \) and of GTP hydrolysis being ~3.5 \( \text{min}^{-1} \). However, the huge difference (about 700 times) in the rates obtained in these two conditions indicates that neither GTP hydrolysis nor Pi release is the rate limiting step in the multiple turnover reaction. Instead, it suggests that the EF-Gs use the same average time for recycling on the naked ribosome.

In line with the tripeptide experiments, multiple turnover GTP hydrolysis with the WT and the M16I EF-Gs were reduced to ~20 % in the presence of FA (0.5 mM), while the FA resistant EF-Gs F88L and F88L+M16I remained essentially unaffected (Figure 5C).

**Overall structures of the S. aureus EF-G mutants:**

*S. aureus* EF-G mutants F88L, F88L+M16I and M16I were crystallized under conditions identical to WT and seeded from WT crystals. The structures were solved by molecular replacement using domains of the WT structure (22). The refined structures at 3.1, 3.1 and 3.0 \( \text{Å} \) resolution, respectively, contain two molecules in the asymmetric unit; all with residues 2-36, 65-400, 404-442, 447-497 and 505-691 ordered. The switch I region (residue 37 to 64), was disordered in all mutant structures, as in previous crystal structures of EF-G on or off the ribosome (for example (13,20,40-43)) except WT *S. aureus* EF-G, where it is partially ordered (22).

Despite similar crystal contacts in the same space group and seeding from WT crystals, the size of the unit cell for the mutant EF-G crystals was dramatically different from the WT (Table S1). The overall conformation of all three EF-G mutants differed significantly from the WT structure (22) in the orientation of domains IV-V with respect to domains I and II. The mutant conformations are also distinct from previous structures of *T. thermophilus* EF-G on and off the ribosome (Figure S2). This conformational change mainly occurs around the hinge connecting domains I–III to IV-V and leads to movement of the tip of domain IV by 5.8 \( \text{Å} \) in M16I, 7.9 \( \text{Å} \) in F88L and 9.2 \( \text{Å} \) in M16I+F88L structure compared to WT (Figure 6A, S2). The same hinge is involved in the larger conformational change between free and ribosome-bound EF-Gs (Figure S2).

**F88 and the switch II region:**

The strictly conserved F88 residue is located in the switch II region of domain I of EF-G, surrounded by domains II, III and V. Switch II has a similar conformation in all three mutant structures that is different from its conformation in WT *S. aureus* EF-G (Figure 6B). While in the WT structure F88 contacts domain V, in all the mutants the loop formed by residues 84 to 90 shows a shift of approximately 5 \( \text{Å} \) so that the side chain of the residue 88 contacts domain III (Figure S3). This contact pushes domain III 2.5-2.6 \( \text{Å} \) away from domain I (Figure S3A) in a direction away from the linker between domains II and III (residue 400 – 406). This leads to an exposed linker that now becomes disordered. In the mutants, residue 88 makes hydrophobic interactions with residues L456 and I460 in helix BIII that line the FA binding site (13) and contains several FA resistant mutations (4,22,44,45). In the F88L and M16I+F88L structures, the smaller leucine side chain in position 88 allows domain III to come 1.6 \( \text{Å} \) closer to domain I compared to the M16I structure, while similar hydrophobic interactions are maintained (Figure S3B-C).

The altered switch II conformation also induces a large shift of domain V with respect to domain I,
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in the direction of the helices in domain V (Figure 6A). In M16I, domain V is shifted by 16 Å with respect to domain I and for F88L and M16I/F88L the corresponding shift is 8 Å. The hidden surface area is dramatically decreased in the domain I-V interaction from 582 Å² in the WT structure (22) to 299 Å² in M16I, 247 Å² in F88L+M16I and 224 Å² in F88L. Also, in M16I and the WT, there are stabilizing inter-domain hydrogen bonds between domain I and domain V that are not present in the other two mutants. While between WT and *T. thermophilus* EF-G structures (20,22) the interface between domains I and V is close to identical, there is a 7 Å movement in a similar direction as observed in the mutants to the FA-locked EF-G conformation on the ribosome (13). Interestingly, the surface of domain V facing domain I has several sites of FA resistance mutations (reviewed in (22)).

Next to F88, residue D87 is involved in interactions that stabilize the inter-domain interface. In the WT EF-G structure, D87 makes a salt bridge to R659 in domain V, while D87 in M16I instead points towards the backbone amide of position 670 and in F88L it towards Q115 in domain I (Figure 6B). Our observations clearly demonstrate that F88 can trigger global conformational changes of EF-G.

**M16 and the hydrophobic core of domain I:**

M16 is a non-conserved residue within a block of conserved residues at the end of a beta strand just before the P loop in domain I. In the *S. aureus* WT structure (22), this residue is located in a hydrophobic core formed by residues L96, L99, A102, V119, A123 and V128 (Figure 6C, S4). This core seems to be a hub for the inter-domain interactions of domain I. Apart from M16, it comprises residues from helix C_{6} that follows switch II in the sequence and packs against domain II (L96 and L99), from helix D_{6} that packs against domain V (V119 and A123) and from switch II (T82 that makes a hydrogen bond to the backbone carbonyl of I15). The side-chains in positions 16, 96 and 123 come together from three corners of a triangle in this core. The amino acids in these three positions are not conserved, but seem to have compensated each other in size and shape during evolution. Species lacking methionine in position 16 (*e.g. T. thermophilus*) instead have larger side chains in positions equivalent to 96 or 123 (Figure 6C, S4).

Upon mutation of M16 to a smaller residue I in the mutants M16I and F88L+M16I, only a small shift in the backbone conformation was observed with reduced distances between the C_{α} atoms compared to the WT and F88L mutant respectively. But, the distances of hydrophobic interactions with the surrounding residues (L96 and L99 from helix C_{6}) notably increase (Figure 6C), suggesting a destabilizing or loosening effect of the hydrophobic core. The F88L mutation also showed small changes in the core compared to the WT, but in this case distances between the C_{α} atoms were increased. This core is clearly a link between switch II conformation and interactions between domain I and II. As discussed later, we propose that these changes are the reason for improved function of the M16I mutation both in WT and F88L background.

**DISCUSSION**

Ribosome interactions with EF-G and mechanism of translocation.

Despite of availability of the structures of several ribosome-EF-G complexes, the precise mechanism of tRNA translocation remained elusive. EF-G has been visualized with GDPNP on ratcheted 70S ribosomes (16), and with GDP and fusidic acid in intermediate states of ratcheting (46) as well as in the classical, non-ratcheted state (13,47). However, there is still no structure of EF-G in a pre-translocation state with a peptidyl-tRNA in the A site.

EF-G interacts with the ribosome through some definite sites (Figure 6D). These include the interactions of 23S rRNA and L6 with domain V, of L12-CTD and SRL with domain I and of 16S RNA with domain II of EF-G. Besides these, there are temporary interactions such as 16S rRNA with switch I in the GDPNP state (16), S12 with domain III and the A-site interactions of domain IV in the post-translocation state (13). Similar ribosomal interactions are possibly present in the pre-translocation state too, perhaps in combination with other transient interactions.
The currently accepted model of translocation is that a conformational change in EF-G after GTP hydrolysis (48) together with back-ratcheting of the ribosomal subunits drives tRNA-mRNA movement, although the exact sequence of the events is not fully established (Figure 7) (35,46,49,50). Most likely there is interplay between conformational changes in EF-G and the ribosomal ratcheting through ribosome – EF-G interactions (16). Thus, one can assume that a defect in the conformational change in EF-G would also influence the down-stream processes such as back ratcheting and tRNA-mRNA movement.

Pivotal role of F88 in triggering conformational changes in EF-G.

Comparisons of the crystal structures of EF-G on and off the ribosome demonstrate the dynamic nature of switch II including F88 (13,22,43). It has been suggested that F88 plays a pivotal role in transmitting the conformational change of switch II to other domains of EF-G in a sequential manner so that EF-G acquires the conformation needed for tRNA translocation (19). This ability of F88 to induce conformational change in EF-G is clearly demonstrated in the structures of mutant *S. aureus* EF-Gs. Compared to the WT, where F88 interacts with domain V, the side chain of F/L88 show interaction with domain III instead in the mutants, causing a major shift of the domain V with respect to domain I (Figure 6A). Similar movement of the F88 equivalent between the domain I-III and I-V interfaces has been observed in structures of *T. thermophilus* EF-G as well (20,42), although without major conformational changes most likely due to crystal packing.

F88 acts as a molecular switch that senses and coordinates the conformational changes between various domains of EF-G necessary for successful translocation and, presumably, also for ribosome recycling. Therefore, although F88L shows no defect in GDP or GTP binding (Figure 4, Table 2) or in GTP hydrolysis (Figure 5), its deficiency in triggering the required conformational change in EF-G leads to a significantly slower translocation compared to the WT (Figures 7, 2A, Table 1).

The slow translocation with F88L causes significant drop-off of the dipeptidyl-tRNA from the A-site even in the single turn-over condition (Figure 2D). However, the drop-off can also be due to poor stabilization of the ratcheted state of the ribosome with F88L, since F88 has been suggested to contribute to stabilizing the ratcheted state of the ribosome in the GDPNP state (16). The defects in translocation and drop-off amplify in every round of peptide elongation and make protein synthesis highly defective. This explains why no luciferase synthesis could be detected in the RTTF assay using F88L (Figure 1B).

**F88L and FA resistance: correlation to fitness.**

The F88 residue makes a direct contact with FA as seen in the crystal structure of FA trapped EF-G on the ribosome (13). Although it is not clear whether the aromatic ring of F makes stacking interaction with FA or not, it certainly requires proper positioning of switch-II. Thus, mutation of F88 to L, with a smaller side chain lacking the aromatic ring, will primarily result in the loss of the contact between residue 88 and FA. Furthermore, it may also alter the overall shape of the FA binding pocket in the inter-domain space of EF-G. These alterations probably reduce the affinity of the ribosome-bound EF-G for FA or result in faster dissociation of FA due to unstable binding (Figure 7). Failing to lock EF-G on the ribosome will allow EF-G to dissociate from the ribosome; thereby rendering resistance to FA. This is evident from our nitrocellulose filter binding experiment where the F88L mutants were defective in forming a stable complex with FA on the ribosome (Figure 1A). Since F88L and the F88L+M16I EF-Gs were both highly similar to the WT in their affinities towards GTP and GDP as well as in ribosome stimulated GTP hydrolysis (Figure 4, 5) we can rule out that FA resistance would arise from deficiency of the F88L mutants in GTP / GDP binding or in GTP hydrolysis.

From a simple comparison of F88L, which is defective in function and resistant to FA, with M16I that shows the highest activity and hypersensitivity to FA, it may seem that there is a direct correlation between fitness (owing from EF-G activity in translocation and recycling) and FA sensitivity. However, this is probably not the case since the fitness-compensatory mutant F88L+M16I, although better than F88L in translocation and...
recycling shows much higher resistance (IC$_{50}$ ~1000 µM) than all other EF-Gs including F88L (IC$_{50}$ ~300 µM) (Figure 2C). The varying resistance between F88L and F88L+M16I signifies the importance of overall geometry of the FA binding pocket over the individual contribution of F88 residue in FA locking of EF-G. Perhaps the M16I mutation in combination with F88L alters the FA binding pocket in such a way that binding of FA becomes more challenging resulting in much higher resistance.

**Mutation M16I – higher activity and hypersensitivity to FA.**

The M16I mutation was slightly (1.2 times) faster than WT in the tripeptide reaction that involved a single translocation event and also in single round ribosome recycling (Figure 2A, Table 1). These gains were significantly amplified during synthesis of full length firefly-luciferase that involved several rounds of translocation and recycling (Figure 1B). The improved activity of M16I can arise from its higher affinity to GTP; similar to the G16V mutation in *T. thermophilus* (corresponding to G14 in *S. aureus* numbering, Figure S4) (21). Since both of these residues are located in the vicinity of the GTP binding site it is likely that they have implications in GTP binding.

In the structure of M16I, we observe a looser packing of the hydrophobic core in domain I involving residues from helix C$_G$ (residue 90-98) that packs against domain II. A similar effect was also observed in the G16V mutant in *T. thermophilus* where both switch II and helix C$_G$ were pushed away from V16. It seems that the loosening of the core alters the conformational dynamics of EF-G directly or through effects on the domain I-II interface, thereby facilitating translocation and ribosome recycling.

Coincidently, both M16I (*S. aureus*) and G16V (*T. thermophilus*) mutations are hypersensitive to FA. Perhaps the dynamic properties of these EF-G mutants that facilitate translocation also favor FA binding to these mutants.

**Fitness compensation in F88L with an additional mutation M16I.**

As discussed above, a loosening of the hydrophobic core in M16I favors conformational dynamics in EF-G. This is also applicable in the background of the F88L mutation. Thus, in the double mutant F88L+M16I, L88 can substitute F88 more efficiently in triggering conformational change (Figure 7). This in turn allows F88L+M16I mutant to be more efficient than F88L in tripeptide formation and ribosome recycling. Moreover, in the double mutant, L88 may also form the required interaction with switch I, stabilizing the ratcheted ribosome and leading to significant decrease in peptidyl tRNA drop-off, together explaining its improved fitness *in vivo.*

**Comparison with other fitness-compensatory mutations.**

Several other fitness-compensatory mutants to F88L were identified in the same study as M16I (4). Out of these, A70V introduces a larger side chain in the hydrophobic core of domain I while A66S, A67V, V284F and I287M/T all introduce changes at the interface between domains I and II (Figure 6D). Just like M16, A70 contacts side chains from helix C$_G$ that packs against domain II. In another study, starting with the primary FA resistance mutation P413L in the linker between domain II and III (equivalent to P406 in *S. aureus*), fitness compensatory mutants of *Salmonella typhimurium* EF-G were selected (1). Interestingly, despite a primary mutation outside the FA binding residues, some of the fitness-compensatory mutations occurred at the same sites as for F88L (4). These mutations in residues 67, 284 and 287 (*S. aureus* numbering) are all likely to disturb the packing between domains I and II.

In summary, the ribosomal interactions involving mainly the contacts of domains I and II may limit the possible conformations of switch II so that L88 cannot substitute effectively for F88 in the F88L mutation, resulting in slow translocation and ribosome-recycling leading to fitness cost. A loosening of the hydrophobic core in domain I and altered interaction between domains I and II in the fitness compensatory mutants (especially in case of M16I) may allow switch II to reach conformations where L88 interacts more efficiently with switch I and domains III and V making EF-G more efficient in translocation and recycling and thereby improving fitness.
Fusidic acid resistance and fitness in S. aureus

REFERENCES

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Activity of the S. aureus EF-Gs in 70S-EF-G-GDP-FA complex formation and luciferase synthesis in RTTF system: Bar diagrams showing relative amounts of (A) 70S-EF-G-GDP-FA complex formed by incubation of 70S ribosomes, [3H]GTP and FA with EF-G at 37°C and (B) firefly-luciferase synthesized in the RTTF system. In B, filled and unfilled bars are without and with FA (250 µM) respectively. In both the experiments, the highest reading obtained was normalized to 100 %.

Figure 2. Activity of the S. aureus EF-Gs in tripeptide formation without or with FA: (A) Time-course of tripeptide (fMet-Phe-Phe) formation with S. aureus EF-Gs; WT (1), M16I (2), F88L (3) and F88L+M16I (4) in the absence and (B) in the presence of FA (250 µM). (C) Bar-diagram showing the amount of tripeptide formed with different EF-Gs with increasing concentration of FA. The concentration of FA corresponding to the midpoint of the transition denoted IC50. In experiments A, B and C, 100 % represents the highest yield of tripeptide. (D) Bar diagram presenting measurements of peptidyl tRNA drop-off during tripeptide reaction with EF-G variants in absence (filled bars) and presence of FA (250 µM) (unfilled bars) considering total [3H]fMet-tRNAfMet as 100 %.

Figure 3. Activity of the S. aureus EF-Gs in ribosome recycling: Splitting of a post-termination complex containing 70S ribosome programmed with MFF mRNA and deacylated tRNA, by the action of RRF and EF-G variants WT (1), M16I (2), F88L (3) and F88L+M16I (4). The reactions were followed in a stopped flow apparatus by monitoring Rayleigh light scattering (at 430 nm) (average of three independent experiments).

Figure 4. GTP and GDP binding to the EF-G variants from S. aureus: Association of (A) mant-GDP and (B) mant-GTP to the S. aureus EF-Gs followed by increase in mant fluorescence at 445 nm (normalized to 1.0). The apparent rates (kapp) derived from the binding experiments were plotted against the concentration of mant-GDP (C) and mant-GTP (D) Each point is a mean of minimum three experiments. The kon and koff were derived from the slopes and the Y intercepts of the respective plot. Panels (E) and (F) represent chase experiments where mant-GDP (E) and mant-GTP (F) bound to EF-Gs were rapidly exchanged with unlabeled nucleotides. The koff values were determined from the rate of decrease of mant fluorescence. In
all these plots (average of minimum three independent measurements) we present WT in grey, M16I in blue, F88L in red and M16I + F88L in green.

**Figure 5.** GTP hydrolysis with the *S. aureus* EF-Gs. (A) Single turnover GTP hydrolysis studied by increase in MDCC-PBP fluorescence upon Pi release, where EF-Gs (2 µM) were rapidly mixed with 70S ribosome (0.5 µM) and GTP (1 mM) in a stopped-flow instrument. The burst phase was used to estimate the rate of single turnover reaction. (B) Multiple turnover GTP hydrolysis followed in TLC by mixing EF-Gs (1 µM) with 70S ribosomes (4 µM) and [3H]GTP (10 µM). The reactions were quenched at different time points and [3H]GDP produced was estimated from the relative counts in the respective band. (C) The effect of FA (500 µM) (unfilled bars) on multiple turnover GTP hydrolysis (10 min) with EF-Gs.

**Figure 6.** Structural comparisons of WT and mutant EF-Gs. (A) The *S. aureus* mutant EF-G structures M16I in blue, F88L in red and M16I+F88L in green are superimposed on the WT structure in grey (pdb 2xex) (22), based on domain I and II (residue 2-400). Arrows indicate the directions of main conformational changes including the movement of the tip of domain IV by 5.8 Å, 7.9 Å and 9.2 Å and shift of Domain V by 16 Å, 8 Å and 8 Å in M16I, F88L and M16I+F88L, respectively, relative to the wild type. (B) The switch II region and its interactions with domain V of EF-G. Hydrogen bonds are shown as dashed lines. Colors as in A. (C) Residue M16 and the hydrophobic core of domain I. *T. thermophilus* EF-G (1fnm) (20) is shown in yellow, other colors as in A. Van der Waals interactions are shown as dashed lines. (D) Ribosomal contacts and sites of compensatory mutations to F88L. The domains of WT *S. aureus* EF-G (22) are shown by colored surface mesh (domain I blue, domain II yellow, domain III green, domain IV red, domain V orange). Ribosomal contacts were compiled from structures of EF-G on the ribosome (13,16,46). Solid lines indicate contacts that are present in all structurally characterized states of EF-G-ribosome complexes while dashed lines indicate contacts only present in some state(s). F88 is shown in blue and sites of fitness-compensatory mutations are shown in magenta.

**Figure 7.** Kinetic scheme of translocation showing the steps where F88L mutants are impaired. A pre-translocation complex in ratcheted conformation (in blue) with tRNAs in A/P and P/E state (I) with EF-G-GTP (red) bound to it undergoes GTP hydrolysis resulting into EF-G-GDP (yellow) (II). Then EF-G undergoes conformational change (orange) (III), which together with back-ratcheting of the ribosome leads to tRNA-mRNA movement relative to 30S. This step commonly called as translocation, brings back the ribosome in a classical non-ratcheted state with tRNAs in P/P and E/E state (IV). Next, either EF-G-GDP releases from this state (V) or gets locked with FA (VI). As indicated with the red oblique lines, the F88L mutants are defective in EF-G conformational change (between stages II and III) resulting in defective translocation and hence showing fitness defect. Similarly, red oblique lines between the states IV and VI indicate that these mutants cannot be locked with FA. The solid lines indicate higher degree of defect than the dashed line.
### Table 1.
The rates of tripeptide formation with *S. aureus* EF-Gs in absence or presence of FA (250 µM) (average of minimum three independent measurements expressed with standard error of the mean (51)).

<table>
<thead>
<tr>
<th>EF-G</th>
<th>Without FA (s⁻¹)</th>
<th>With FA (s⁻¹)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.67 ± 0.023</td>
<td>0.037 ± 0.002</td>
<td>18 times</td>
</tr>
<tr>
<td>M16I</td>
<td>0.84 ± 0.028</td>
<td>0.019 ± 0.001</td>
<td>40 times</td>
</tr>
<tr>
<td>F88L</td>
<td>0.07 ± 0.004</td>
<td>0.07 ± 0.03</td>
<td>none</td>
</tr>
<tr>
<td>F88L+M16I</td>
<td>0.21 ± 0.015</td>
<td>0.23 ± 0.04</td>
<td>none</td>
</tr>
</tbody>
</table>

### Table 2.
Affinity of *S. aureus* EF-Gs to GTP and GDP. The $k_{on}$ and $K_D$ values are from the plots in Figure 4C and D. The $k_{off}$ values are from the chase experiments (Figure 4E, F). All are average of minimum three independent measurements expressed with standard error of the mean (51).

<table>
<thead>
<tr>
<th>EF-G</th>
<th>$k_{on}$-$\text{mantGDP}$ (µM⁻¹ s⁻¹)</th>
<th>$K_D$-$\text{mantGDP}$ (µM)</th>
<th>$k_{off}$-$\text{GDP}$ (s⁻¹)</th>
<th>$k_{on}$-$\text{mantGTP}$ (µM⁻¹ s⁻¹)</th>
<th>$K_D$-$\text{mantGTP}$ (µM)</th>
<th>$k_{off}$-$\text{GTP}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.5 ± 0.4</td>
<td>27 ± 2</td>
<td>206 ± 3.6</td>
<td>3.1 ± 0.4</td>
<td>81 ± 12</td>
<td>288 ± 2.7</td>
</tr>
<tr>
<td>M16I</td>
<td>6.3 ± 0.6</td>
<td>25 ± 3</td>
<td>184 ± 3.5</td>
<td>0.9 ± 0.1</td>
<td>63 ± 10</td>
<td>97 ± 3.2</td>
</tr>
<tr>
<td>F88L</td>
<td>6.8 ± 0.3</td>
<td>24 ± 1</td>
<td>183 ± 2.5</td>
<td>2.9 ± 0.3</td>
<td>90 ± 11</td>
<td>278 ± 1.4</td>
</tr>
<tr>
<td>F88L+M16I</td>
<td>6.1 ± 0.3</td>
<td>24 ± 1</td>
<td>158 ± 2.2</td>
<td>2.5 ± 0.1</td>
<td>77 ± 5</td>
<td>214 ± 2.6</td>
</tr>
</tbody>
</table>
Fusidic acid resistance and fitness in S. aureus

Figure 1

A

% Complex formed

WT  M16I  F88L  F88L+M16I  -70S  -EF-G  -FA

B

% Luciferase activity

WT  M16I  F88L  F88L+M16I
Figure 2

A. - FA

B. + FA

C. Tripeptide MFF (%) vs. Conc. of FA (mM)

D. tRNA drop-off (%)
Figure 3

![Graph showing light scattering over time with different markers for WT, M16I, F88L, and F88L+M16I](image-url)
Fusidic acid resistance and fitness in S. aureus

Figure 4

A

B

C

D

E

F

Mant-GDP fl. (a.u.)

Mant-GTP fl. (a.u.)

Mant-GDP fl. (a.u.)

Mant-GTP fl. (a.u.)

Mant-GDP fl. (a.u.)

Mant-GTP fl. (a.u.)

WT

M16I

F88L

F88L+M16I

WT

M16I

F88L

F88L+M16I

WT

M16I

F88L

F88L+M16I

WT

M16I

F88L

F88L+M16I

WT

M16I

F88L

F88L+M16I

WT

M16I

F88L

F88L+M16I

WT

M16I

F88L

F88L+M16I

Time (sec)

Time (sec)

Time (sec)

Time (sec)

Mant-GDP (μM)

Mant-GTP (μM)

K_{app} (s^{-1})

K_{app} (s^{-1})

K_{app} (s^{-1})

K_{app} (s^{-1})

0.00 0.02 0.04 0.06 0.08 0.10

0.00 0.02 0.04 0.06 0.08 0.10

5 10 15 20

10 20 30 40 50

0.00 0.02 0.04 0.06 0.08 0.10

0.00 0.02 0.04 0.06 0.08 0.10

0.00 0.02 0.04 0.06 0.08 0.10

0.00 0.02 0.04 0.06 0.08 0.10

Mant-GDP (μM)

Mant-GTP (μM)
Figure 5

A

Pl release / EF-G

WT
M16I
F88L
F88L+M16I

Time (sec)

B

% GTP hydrolysed

WT
M16I
F88L
F88L+M16I

Time (min)

C

% GTP hydrolysed

WT
M16I
F88L
F88L+M16I

% GTP hydrolysed

0
25
50
75
100
Fusidic acid resistance and fitness in S. aureus

Figure 7

(I) 50S EF-G

30S GTP hydrolysis

(II) EF-G conformational change

F88L F88L+M16I

(III) Back ratcheting tRNA-mRNA movement

(EF-G release)

(IV) FA locking

(V)

(F88L+M16I)

(VI)
Mechanism of elongation factor-G mediated fusidic acid resistance and fitness compensation in *Staphylococcus aureus*

Ravi Kiran Koripella, Yang Chen, Kristin Peisker, Cha San Koh, Maria Selmer and Suparna Sanyal

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