Mechanism of Elongation Factor-G-mediated Fusidic Acid Resistance and Fitness Compensation in Staphylococcus aureus

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Background: Fusidic acid-resistant EF-G mutants of Staphylococcus aureus show fitness loss and compensation.

Results: Slower translocation and ribosome recycling from restricted conformational change, plus increased tRNA drop-off, cause fitness loss in F88L, which are recovered in F88L/M16I, leading to fitness compensation.

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

Significance: This work clarifies how antibiotic-resistant mutations affect in vivo fitness.

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 defects 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 Phe-88 in switch II is a key residue for FA locking and also for triggering interdomain 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 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 a 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. Because 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...
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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 at its tip, which is Phe-88 in *S. aureus* and Phe-90 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 Phe-90 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 fitness loss (21) through an unknown molecular mechanism.

In this study, we have characterized, with steady-state and kinetic assays, 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 cross-talk between different residues in EF-G influences its function and provides insights into the molecular mechanism of FA resistance, fitness loss, and fitness 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 Leu-88 to Phe in the double mutation construct F88L/M16I using the QuikChange mutagenesis kit (Qiagen). Overexpression 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 *Escherichia coli*. MRE600 ribosomes, His-tagged translation factors, XR7 fMet-Phe-Phe-stop (MFF) mRNA and fMet-tRNA^fMet^ were purified as shown by Mandava *et al.* (24) and Huang *et al.* (23). 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 (10 mM), pyruvate kinase (50 μg/ml), and myokinase (2 μg/ml), resulting in a final magnesium concentration of ~1.5 mM. FA was obtained from Leo Pharma (Denmark). tRNA^Phe^ was purchased from Chemical Block (Moscow, Russia). Phosphoenolpyruvate, pyruvate kinase, myokinase, and nonradioactive amino acids were from Sigma-Aldrich. Radioactive amino acids and nucleotides triphosphates were from GE Healthcare.

70S EF-G-GDP-FA Complex Formation—1 μM 70S ribosome, 5 μM EF-G, 10 μM [3H]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 min 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 [3H]GDP remaining in the filter indicated the amount of the 70S EF-G-[3H]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 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*_app) were estimated by fitting the curves with single exponential using Origin 8.0 program. Linear fitting of *k*_app values against the concentration of mant-GDP/GTP resolved into a straight line. Association and dissociation rate constants, *k*_on and *k*_off were determined from the slope of the plot and the y axis intercept, respectively. The equilibrium dissociation constant (*K*_D) was estimated from *k*_off/*k*_on.

Dissociation Experiments—The dissociation rate constant, *k*_off, was also estimated directly by chasing the 4 μM mant-GDP/GTP in complex with 2 μM EF-G with 400 μM unlabelled nucleotides in a stopped flow apparatus. The curves were fitted with single exponential using Origin 8.0.

**GTP Hydrolysis and P 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 [3H]GTP. The reactions, quenched at different time points with 50% formic acid, were analyzed in with thin layer chromatography (TLC). The extent of GTP hydrolyzed at each time point was measured from the ratio of [3H]GDP to the total count of [3H]GTP plus [3H]GDP, and the rates were determined by fitting the data using single exponential function.

GTP hydrolysis under single turnover condition was measured from P<sub>i</sub> release, monitored by the increase in fluorescence of MDCC-labeled phosphate-binding protein MDCC-PBP (gift from Prof. Martin Webb, Medical Research Council, London, UK) in a stopped flow instrument as described in Refs. 23 and 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 37°C, and the fluorescence change with time was recorded using a 475-nm long-pass filter. The rate of P<sub>i</sub> release was obtained by fitting the initial burst phase with a single exponential.

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

**Peptidyl tRNA Hydrolase Assay**—Peptidyl tRNA hydrolase is an enzyme specific for ester bond hydrolysis of the peptidyl tRNAs off the ribosome. 100 μM peptidyl tRNA hydrolase was added to the tripeptide reaction described above. After 3 min, 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 <sup>3</sup>H count in the supernatant with the total count.

**Ribosome Recycling**—A termination complex containing 70S ribosomes (0.5 μM), XR7 mRNA: fMet-Phe-Leu-stop (2 μM), tRNA<sup>Fhe</sup> (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 polyxim at 37°C. 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 the addition of pET30-Luc DNA and was followed by increase in luminescence measured in a GloMax 20/20 luminometer for 60 min at 37°C 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, The European Synchrotron Radiation Facility (ESRF, Grenoble, France), with an ADSC Quantum Q315r detector at 100 K and 0.954 Å. The data were processed and scaled with the XDS package (25). The crystals belong to space group P2<sub>1</sub>, x-ray data statistics are summarized in supplemental Table S1. The structures were solved by molecular replacement with PHASER (26), using domain I and II of WT EF-G (Protein Data Bank 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 supplemental Table S1. Sequence alignment was performed using ClustalW (31). Structure superposition was performed with O (32). All of the structure figures were generated using PyMOL (PyMOL Molecular Graphics System, version 1.2r3pre; Schrödinger).

**Protein Data Bank Codes**—The refined structures were deposited in the Protein Data Bank in Europe with the Protein Data Bank codes of 3ZZO (M16I), 3ZZT (F88L), and 3ZZU (F88L/M16I).

**RESULTS**

**Comparison of the S. aureus EF-G Mutants in Ribosomal Complex Formation with FA**—In the presence of 70S ribosomes, EF-G hydrolizes GTP and EF-G-DP can be trapped on the 70S ribosome with 0.5 mM FA, resulting in a 70S EF-G-DP-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% of complex was formed with F88L and F88L/M16I mutants, comparable with the background without 70S or EF-G or FA (>10%) (Fig. 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.

**Performance of S. aureus EF-G Mutants in RTTF**—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%), whereas WT EF-G showed a moderate level (~60%) of synthesis (Fig. 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 (Fig. 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 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 MFF formation assay where an initiation complex containing 70S ribosome programmed with MFF coding mRNA and fMet-tRNA<sup>Fmet</sup> was rapidly mixed in quench flow with an elongation mix containing EF-Tu, Phe-tRNA<sup>Phe</sup>, and EF-G at 37 °C. Under our experimental conditions, the formation of MF dipeptide took ~7ms ($k_{\text{dipep}} = 140$ s<sup>−1</sup>) (supplemental Fig. 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 as 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 Fig. 2A, the rate of tripeptide formation using WT *S. aureus* EF-G was $0.67 \pm 0.023$ s<sup>−1</sup>. The reaction was faster ($k_{\text{tripep}} = 0.84 \pm 0.028$ s<sup>−1</sup>) with the M16I mutant. Compared with these two, the FA-resistant mutant F88L was significantly slower ($k_{\text{tripep}} = 0.07 \pm 0.004$ s<sup>−1</sup>). However, the double mutant F88L/M16I was three times faster than F88L ($k_{\text{tripep}} = 0.21 \pm 0.015$ s<sup>−1</sup>), 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 (Fig. 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.

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The M16I mutation displayed the highest decrease, indicating hypersensitivity of this mutant toward 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 (Fig. 2C). Although IC$_{50}$ for the WT was 100 μM, it was lower for M16I (~50 μM), indicating its higher sensitivity to FA. As expected, F88L showed a higher IC$_{50}$ value (~300 μM). Surprisingly, F88L/M16I showed a markedly higher IC$_{50}$ of ~1000 μM. This was somewhat unexpected because 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/M16V mutant behaved very much like the F88L/M16I mutant and produced a IC$_{50}$ value of ~1000 μM (supplemental Fig. S1B).

**Peptidyl tRNA drop-off—**One notable feature of the F88L mutant was the lower yield of tripeptide (60%) compared with the other EF-Gs irrespective of the absence or presence of FA (Fig. 2, A and 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 peptidyl tRNA hydrolase, which cleaves off peptides only from free peptidyl-tRNAs outside the ribosome.

As expected, ~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 (Fig. 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 (Fig. 2B and 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 in Ribosome Recycling—**EF-G has a second function in dissociation of the post-termination ribosomes into subunits, so-called “ribosome recycling” together with RRF (36–38). The subunit dissociation with different EF-Gs was monitored by Rayleigh light scattering in a stopped flow instrument (Fig. 3). Consistent with their performance in the tripeptide assay, the F88L mutant produced the slowest rate of subunit dissociation ($k_{\text{recycle}} = 0.008 \pm 0.001 \text{ s}^{-1}$), showing that this mutant is defective in the ribosome recycling step as well. In comparison, the fitness compensatory mutant F88L/M16I was ~3-fold faster ($k_{\text{recycle}} = 0.021 \pm 0.001 \text{ s}^{-1}$) in recycling ribosomes. The WT and M16I mutant were 20 and 22 times faster than F88L and dissociated the ribosomes at rates of 0.16 ± 0.012 and 0.18 ± 0.007 s$^{-1}$, respectively. Because at higher concentrations, FA forms micelles, 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 toward GDP and GTP was estimated using mant-GDP/GTP (39). Fig. 4 (A and B) illustrates the association experiments with *S. aureus* EF-Gs, where the increase in mant fluorescence upon mant-GDP/GTP binding was monitored in stopped flow. The apparent rates ($k_{\text{app}}$) derived from the binding experiments showed linear concentration dependence for mant-GDP (Fig. 4C) and mant-GTP (Fig. 4D). The association rate constants ($k_{\text{on}}$) and the equilibrium dissociation constants ($K_d$) for mant-GDP and -GTP are summarized in Table 2. All EF-G variants were highly similar in GDP binding with $k_{\text{on}} = \sim 6 \text{ μM}^{-1} \text{ s}^{-1}$ and $K_d_{\text{mantGDP}} = \sim 25 \text{ μM}$ (Fig. 4C). In contrast, some variation in $K_d_{\text{mantGTP}}$ was seen for the EF-G variants in GTP binding (Fig. 4D). The FA-resistant, fitness-compromised mutant F88L showed lowest affinity for GTP (90 μM), whereas the FA hypersensitive, high activity mutant M16I showed the 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 (Fig. 4, E and F). In all cases, these values matched the ones determined from the $y$ intercept of the plots in Fig. 4 (C and D). Although $K_{\text{d-mantGDP}}$ 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 s$^{-1}$) (Fig. 4, D and F), contributing to its low $K_{p_{\text{mantGTP}}}$—putting these results together, the affinity to GTP (but not to GDP) seems to have a correlation with the activity of the EF-Gs.

**TABLE 1**

<table>
<thead>
<tr>
<th>EF-G</th>
<th>Without FA ($s^{-1}$)</th>
<th>With FA ($s^{-1}$)</th>
<th>Fold decrease</th>
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<tr>
<td>WT</td>
<td>0.67 ± 0.023</td>
<td>0.017 ± 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>

**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 (point 1), M16I (point 2), F88L (point 3), and F88L/M16I (point 4). The reactions were followed in a stopped flow apparatus by monitoring Rayleigh light scattering (at 430 nm) (average of three independent experiments).
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 (Fig. 5, A and B), the rate of Pi release being \(45 \text{ ms}^{-1}\) and GTP hydrolysis being \(3.5 \text{ min}^{-1}\). However, the huge difference (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), whereas the FA-resistant EF-Gs F88L and F88L/M16I remained essentially unaffected (Fig. 5, C).

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 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 (residues 37–64), was disordered in all of the mutant structures, as in previous crystal structures of EF-G on or off the ribosome (for example, Refs. 13, 20, and 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 (supplemental Table S1). The overall conformation of all three EF-G mutants differed significantly from the WT structure (22) in the orientation of domains IV and 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 (supplemental Fig. S2). This conformational change mainly occurs around the hinge connecting domains I–III to IV and V and leads to movement of the tip of domain IV by 5.8 Å in M16I, 7.9 Å in F88L, and 9.2 Å in M16I/F88L structure compared with WT (Fig. 6A and supplemental Fig. S2). The same hinge is involved in the larger conformational change between free and ribosome-bound EF-Gs (supplemental Fig. S2).

Phe-88 and the Switch II Region—The strictly conserved Phe-88 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 (Fig. 6B). Although in the WT structure Phe-88 contacts domain V, in all the mutants the loop formed by residues 84–90 shows a shift of ~5 Å so that the side chain of the residue 88 contacts domain III (supplemental Fig. S3). This contact pushes domain III 2.5–2.6 Å away from domain I (supplemental Fig. 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 Leu-456 and Ile-460 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 Å closer to domain I compared with the M16I structure, whereas similar hydrophobic interactions are maintained (supplemental Fig. S3, B and C).

The altered switch II conformation also induces a large shift of domain V with respect to domain I, in the direction of the helices in domain V (Fig. 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 interdomain hydrogen bonds between domain I and domain V that are not present in the other two mutants. Although 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 Ref. 22).

Next to Phe-88, residue Asp-87 is involved in interactions that stabilize the interdomain interface. In the WT EF-G structure, Asp-87 makes a salt bridge to Arg-659 in domain V, whereas Asp-87 in M16I instead points toward the backbone amide of position 670 and in F88L it toward Gln-115 in domain I (Fig. 6B). Our observations clearly demonstrate that Phe-88 can trigger global conformational changes of EF-G.

Met-16 and the Hydrophobic Core of Domain I—Met-16 is a nonconserved residue within a block of conserved residues at the end of a β 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 Leu-96, Leu-99, Ala-102, Val-119, Ala-123, and Val-128 (Fig. 6C and supplemental Fig. S4).
This core seems to be a hub for the interdomain interactions of domain I. Apart from Met-16, it comprises residues from helix CG that follows switch II in the sequence and packs against domain II (Leu-96 and Leu-99), from helix DG that packs against domain V (Val-119 and Ala-123) and from switch II (Thr-82 that makes a hydrogen bond to the backbone carbonyl of Ile-15). 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 for 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 (Fig. 6C and supplemental Fig. S4).

Upon mutation of Met-16 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 Cα atoms compared with the WT and F88L mutant, respectively. However, the distances of hydrophobic interactions with the surrounding residues (Leu-96 and Leu-99 from helix CG) notably increase (Fig. 6C), suggesting a destabilizing or loosening effect of the hydrophobic core. The F88L mutation also showed small changes in the core compared with the WT, but in this case, the distances between the Cα 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 the 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, nonratcheted state (13, 47). However, there is still no structure of EF-G in a pretranslocation state with a peptidyl-tRNA in the A site.

EF-G interacts with the ribosome through some definite sites (Fig. 6D). These include the interactions of 23 S rRNA and L6 with domain V (Val-119 and Ala-123) and from switch II (Thr-82 that makes a hydrogen bond to the backbone carbonyl of Ile-15). 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 for 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 (Fig. 6C and supplemental Fig. S4).

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Pivotal Role of Phe-88 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 Phe-88 (13, 22, 43). It has been suggested that Phe-88 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 Phe-88 to induce conformational change in EF-G is clearly demonstrated in the structures of mutant S. aureus EF-Gs. Compared with the WT, where Phe-88 interacts with domain V, the side chain of Phe/Leu-88 shows interaction with domain III instead in the mutants, causing a major shift of the domain V with respect to domain I (Fig. 6A). Similar movement of the Phe-88 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 caused by crystal packing.

Phe-88 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 (Fig. 4 and Table 2) or in GTP hydrolysis (Fig. 5), its deficiency in triggering the required conformational change in EF-G leads to a significantly slower translocation compared with the WT (Figs. 7 and 2A and 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 (Fig. 2D). However, the drop-off can also be due to poor stabilization of the ratcheted state of the ribosome with F88L, because Phe-88 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 (Fig. 1B).

F88L and FA Resistance: Correlation to Fitness—The Phe-88 residue makes 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 Phe-88 to Leu, 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 interdomain 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 caused by unstable binding (Fig. 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 (Fig. 1A). Because F88L and the F88L/M16I EF-Gs were both highly similar to the WT, 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 downstream processes such as back ratcheting and tRNA-mRNA movement.

FIGURE 7. Kinetic scheme of translocation showing the steps where F88L mutants are impaired. A pretranslocation complex in ratcheted conformation (blue) with tRNAs in A/P and P/E state (stage I) with EF-G-GTP (red) bound to it undergoes GTP hydrolysis, resulting in EF-G-GDP (yellow) (stage II). Then EF-G undergoes conformational change (orange) (stage III), which together with back-ratcheting of the ribosome leads to tRNA-mRNA movement relative to 30 S. This step, commonly called translocation, brings back the ribosome in a classical nonratcheted state with tRNAs in P/P and E/E state (stage IV), which together with back-ratcheting leads to tRNA-mRNA movement relative to 30 S. This step, commonly called translocation, brings back the ribosome. Next, either EF-G-GDP releases from this state (stage V) or gets locked with FA (stage 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 defects. Similarly, the red oblique lines between the states IV and VI indicate that these mutants cannot be locked with FA. The solid lines indicate a higher degree of defect than the dashed line.
Fusidic Acid Resistance and Fitness in S. aureus

in their affinities toward GTP and GDP, as well as in ribosome stimulated GTP hydrolysis (Figs. 4 and 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, which shows the highest activity and hypersensitivity to FA, it may seem that there is a direct correlation between fitness (caused by EF-G activity in translocation and recycling) and FA sensitivity. However, this is probably not the case because the fitness compensatory mutant F88L/M16I, although better than F88L in translocation and recycling, shows much higher resistance (IC50 = 1000 μM) than all other EF-Gs including F88L (IC50 = 300 μM) (Fig. 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 Phe-88 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 (Fig. 2A and Table 1). These gains were significantly amplified during synthesis of full-length firefly luciferase that involved several rounds of translocation and recycling (Fig. 1B). The improved activity of M16I can arise from its higher affinity to GTP, similar to the G16V mutation in *T. thermophilus* (corresponding to Gly-14 in *S. aureus* numbering, supplemental Fig. S4) (21). Because 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_C (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_C were pushed away from Val-16. 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. Coincidentally, 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, Leu-88 can substitute Phe-88 more efficiently in triggering conformational change (Fig. 7). This in turn allows the F88L/M16I mutant to be more efficient than F88L in tripeptide formation and ribosome recycling. Moreover, in the double mutant, Leu-88 may also form the required interaction with switch I, stabilizing the ratcheted ribosome and leading to a 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). Of these, A70V introduces a larger side chain in the hydrophobic core of domain I, whereas A66S, A67V, V284F, and I287M/T all introduce changes at the interface between domains I and II (Fig. 6D). Just like Met-16, Ala-70 contacts side chains from helix C_C 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 Pro-406 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 Leu-88 cannot substitute effectively for Phe-88 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 Leu-88 interacts more efficiently with switch I and domains III and V, making EF-G more efficient in translocation and recycling and thereby improving fitness.

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REFERENCES

Fusidic Acid Resistance and Fitness in S. aureus

Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680


Mechanism of Elongation Factor-G-mediated Fusidic Acid Resistance and Fitness Compensation in *Staphylococcus aureus*

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