Biochemical Characterization of Mutant EF-G from the Fusidic Acid Resistant Strains of *Staphylococcus aureus*

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SUMMARY

*Staphylococcus aureus* is a naturally occurring gram-positive bacterium causing a wide range of illnesses in human. Fusidic acid is one of the potent antibiotics used for the treatment of severe *Staphylococcus aureus* infections. Fusidic acid acts by blocking elongation factor G (EF-G) on the ribosome after tRNA translocation from the A-site to P-site, leading to the inhibition of protein synthesis. Resistance against fusidic acid is caused mainly by mutations in the *fusA* gene encoding elongation factor G. Like other antibiotics fusidic acid resistance is also associated with biological fitness cost in resistant bacteria resulting in reduced growth, impaired virulence and poor survival. With the aim to understand the molecular mechanism of fusidic acid resistance and the fitness compensation mechanism I have chosen in the present study EF-Gs from fusidic acid resistant mutant strains of *Staphylococcus aureus*, consisting of primary fusidic acid resistant mutant (e.g. Phe88Leu) and secondary mutants (Phe88Leu + Met16Val, Phe88Leu + Met16Ile) resulting in fitness compensation in resistant *S. aureus* strains. The *fusA* gene from fusidic acid resistant strains of *S. aureus* has been cloned in a commercial vector for over-expression in *E. coli*. The over-expressed *S. aureus* EF-G proteins were purified using column chromatography and three different types of experiments were conducted for further characterizing of the mutants: (i) nitrocellulose binding assay – to check the capacity of the mutant EF-Gs in fusidic acid binding, (ii) tripeptide formation assay – to test the ability of the mutant EF-Gs in tRNA translocation and (iii) guanidine nucleotide exchange assay – to test the rate of guanine nucleotide exchange by the mutant EF-Gs on and off the ribosome. The filter binding data showed that all mutant EF-Gs conferring fusidic acid resistance bind fusidic acid very poorly. In the first set of mutants (which contained Phe88Leu as the primary mutation) there was no major difference in GDP exchange rate although the exchange rate was slower compared to the wild-type EF-G in the presence of the ribosome. Since both of these features were similar in the primary Phe88Leu as well as in the fitness compensatory mutant EF-Gs from Phe88Leu + Met16Val and Phe88Leu + Met16Ile, they can be attributed to the primary F88L mutation and not to the secondary mutations (Met16Val and Met16Ile). However, the fitness compensatory mutants Phe88Leu + Met16Val and Phe88Leu + Met16Ile showed higher activity in tRNA translocation when compared to the primary mutant Phe88Leu. This activity gain was the direct cause of fitness compensation in these mutants and can be caused by either (i) improved binding of mutant EF-Gs on the ribosome or (ii) by faster GTP hydrolysis.
INTRODUCTION

Bacterial infection

Despite the medical advances during the last century, infectious diseases are still very much of concern to mankind. Bacterial infections cause roughly 50% of the deaths in the world today (Hogberg, 2005). Even though this number represents the world situation, in the western countries bacterial infections have become less eminent due to the introduction of proper antibiotics as well as a better health care system. However, instances of re-emerging diseases due to the development of drug resistance in bacterial species are increasing everyday. *Staphylococcus* infections are examples for this. Therefore, although several antibiotics were useful in controlling *Staphylococcus* infections earlier, only few of them are effective today.

*Staphylococcus aureus*

*Staphylococcus aureus* is one of the naturally occurring common pathogens in human host. It is a gram-positive bacterium, commonly found on moist surfaces such as nasal mucosa, axillae and perineum. Due to the presence of a vast array of virulence factors it can establish infections in every organ in the human body (Lowy, 1998; Sibbald et al., 2006). Although the nosocomial infections are the most common type, *Staphylococcus aureus* can also cause a wide range of illnesses from skin infections to life threatening diseases such as pneumonia and meningitis (Sibbald et al., 2006).

Fusidic acid

![Figure 1. The structure of fusidic acid (Riber et al., 2006).](image)

Fusidic acid is a narrow spectrum steroid antibiotic derived from the fungus *Fusidium coccineum* with a potent activity against gram-positive bacteria, especially against *Staphylococcus aureus* (Besier et al., 2003; Besier et al., 2005; Riber et al., 2006). Since the successful introduction of fusidic acid for the clinical use against *Staphylococcus* infections in 1962, it has continued to draw attention, especially because of the fact that the development of fusidic acid resistance in *Staphylococcus aureus* is much slower than in case of any other antibiotics used before (penicillin and methicillin) (Besier et al., 2003; Besier et al., 2005). Currently fusidic acid is used in the treatment of skin and eye infections, but occasionally it is also used to treat bone infections (Gottlieb et al., 2002)).
How does fusidic acid inhibit bacterial growth?

The molecular target of fusidic acid is elongation factor-G (EF-G) (Hansson et al., 2005; Riber et al., 2006). Fusidic acid inhibits bacterial growth by inhibiting protein synthesis, where it blocks the elongation factor G (EF-G) on the ribosome after tRNA translocation and GTP hydrolysis (Besier et al., 2003; Besier et al., 2005; Bodley et al., 1969; Hansson et al., 2005; Johanson et al., 1996; MacVain et al., 2000; Martemyanov et al., 2001; Nagaev et al., 2001). This phenomenon is also used as an effective tool to understand the mechanistic aspects of translation.

Fusidic acid resistance in *Staphylococcus aureus*

Mechanisms of resistance to fusidic acid in *S. aureus* include 1) target based resistance that includes target gene mutation and target protein protection by structural alteration and 2) unknown resistance that affects bacterial systems other than its direct target EF-G.

Target based resistance

Target-based resistance involves point mutations in the *fusA* gene, coding for EF-G. These mutations cause altered structure and function of EF-G by amino acid substitutions. Many such mutations are clustered in domain III of EF-G (Chopra, 1976; Johanson and Hughes, 1994; Nagaev et al., 2001). Besides these, other mutations are also reported in domain V and I of EF-G even though they are comparatively less effective. *fusA* gene mutations are mostly found in non-epidemic strains of the fusidic acid resistant *Staphylococcus aureus* (O’Neill et al., 2004).

Unknown class of resistance

In the laboratory, *Staphylococcus aureus* strains selected for fusidic acid resistance show mutations mapping outside of the *fusA* gene (Nagaev et al., 2001). How these mutations resulted in fusidic acid resistance is an open question.

Elongation Factor-G and its role in translation

The process of protein synthesis includes three main phases: initiation, elongation and termination, each catalysed by a G-protein factor. These are: initiation factor 2 (IF2), which is required for initiation; elongation factor Tu (EF-Tu), which carries amino-acyl charged tRNAs to the ribosome; elongation factor G (EF-G), which catalyzes tRNA translocation; and release factor 3 (RF3), which releases the class-I release factors after removal of the nascent peptide.

EF-G binds to the translating ribosome when there is a peptidyl tRNA in the A-site and deacylated tRNA in the P-site. Upon binding to the ribosome it changes its conformation, which in turn drives a conformational change on the ribosome where the small subunit rotates with respect to the large subunit of the ribosome (Agrawal et al., 1999; Zavialov et al., 2005). These combined movements translocate the tRNAs at the A- and P-site to the P- and E-sites respectively, while the mRNA advances by one codon (Agrawal et al., 1999; Rodnina et al., 1997). The kinetic studies on translocation suggested that EF-G dependent GTP hydrolysis drives the translocation step (Rodnina et al., 1997), however this concept is highly debated (Zavialov et al., 2005). After the GTP hydrolysis, EF-G-GDP leaves the ribosome for another
cycle of elongation. However, the antibiotic fusidic acid traps EF-G-GDP on the ribosome in the post-translocation state.

**Domain Structure of the EF-G molecule**

EF-G is the translocase of translation, a large GTPase with molecular weight around 80 kDa varying slightly in different bacterial species. It is an elongated molecule consisting of five domains with which it can contact both 30 S and 50 S subunits of the ribosome (Agrawal et al., 1999). Out of the five domains, domain I has two subunits, the GTP binding G domain and a G’ domain, which is absent in most of other translational GTPases. It has been shown that there is a gross structural similarity between the tertiary structures of EF-G and EF-Tu – aminoacyl-tRNA ternary complex (Martemyanov et al., 2001).

![Diagram of EF-G molecule](image)

**Figure 2.** GTP/GDP bound form of EF-G is shown as ribbon, coloured by domain: domain I (G & G’) in blue, domain II- purple, domain III -yellow, domain IV -orange and domain V in green. GTP/ GDP binding site is indicated with an arrow (Adopted from PDB code IFNM).

It has been shown by X-ray crystallography that EF-G in a nucleotide free form is structurally identical to its GTP/GDP bound form off the ribosome (Hansson et al., 2005; Valle et al., 2003). However, cryo-EM studies with functional ribosomes showed that the ribosome bound GTP-form of EF-G is quite different in its domain arrangement from its crystal structures (Valle et al., 2003). These findings together with the biochemical experiments suggest a need of structural flexibility and domain rearrangements to bring out the EF-G function in tRNA translocation and its own release from the ribosome.
**Fusidic acid resistance mutations in fusA gene**

Several fusidic acid resistance mutations that cause amino acid alterations in EF-G have been mapped in the *fusA* gene (Johanson and Hughes, 1994). Since these mutations are spread in different domains of EF-G, it has been suggested that only a few mutations interact directly with the fusidic acid binding site on EF-G while others exert their effect indirectly (Hansson et al., 2005). The mutations believed to hamper fusidic acid binding are mapped at the interface between G domain, domain III and domain V of EF-G (Hansson et al., 2005; Laurberg et al., 2000). The ways these mutations work are not yet well characterised. It has been suggested that either they modify the affinity of EF-G for ribosome or for fusidic acid or for both by restricting the conformational changes required for EF-G function (Hansson et al., 2005). However there is no direct structural or biochemical evidences available in support of these hypotheses.

**Fitness compensatory mutants**

Antibiotic resistance in general is associated with biological fitness cost for the resistant bacteria. Also in *Staphylococcus aureus* such fitness costs for fusidic acid resistance have been found both *in vivo* and *in vitro* (Besier et al., 2005) these are mainly associated with poor growth and other functional abnormalities. However, these costs can be compensated by acquiring additional or secondary mutations that may help the resistant bacteria to grow at a rate much more similar to that of the wild type species (Nagaev et al., 2001). These mutants are called fitness compensatory mutants (FCM).

**Aim**

The aim of the project was to understand the molecular mechanism of fusidic acid resistance in *S. aureus* by biochemical characterization of the EF-Gs derived from the fusidic acid resistant strains of *S. aureus*. My results suggest that the low fitness shown by the primary fusidic acid resistant *S. aureus* strains is due mainly to the inability of its EF-Gs in translocation, which was improved in the strains with fitness-compensatory mutations. This translocation defect may have arisen from lower affinity of the mutant EF-Gs to the pre-translocation state of the ribosome.
RESULTS

Formation of EF-G–fusidic acid complex with ribosomes

Fusidic acid traps EF-G-GDP on the ribosome after GTP hydrolysis. Therefore successful binding of fusidic acid to EF-G-GDP leading to the formation of 70S-EF-G-GDP-FA complex can be monitored and quantified by nitrocellulose filter-binding assay using $[^{3}H]$ counts on $[^{3}H]$GDP. When the 70S-EF-G-$[^{3}H]$GDP-FA complex was formed by incubation of fusidic acid with 70S ribosomes, EF-G and $[^{3}H]$GTP and filtered through a nitrocellulose filter, only larger molecules like ribosome were retained on the nitrocellulose filter. The ribosomes with EF-G-$[^{3}H]$GDP trapped on it with fusidic acid produced a scintillation count that was measured in the scintillation counter.

Wild type and six different mutant EF-Gs from *Staphylococcus aureus* were tested by this assay for their ability to form complexes with fusidic acid (Fig 3). As a negative control, complexes were formed without the addition of EF-G or fusidic acid. Since previous studies suggested that similar complexes can be formed with only the large subunit (50S) of the ribosome (Martemyanov et al., 2001), I repeated the whole set of experiment to check 50S-EF-G-$[^{3}H]$GDP-FA complex formation as well.

Figure 3a shows the data from nitrocellulose filter binding assay with 70S ribosome and figure 3b shows the same with 50S subunit from *S. aureus* ribosome. In both cases it is evident that the ability of the mutant EF-Gs purified from the fusidic acid resistant strains of *Staphylococcus aureus* to bind fusidic acid on the ribosome was very poor compared to that of the wild-type EF-G.
Figure 3. Filter binding assay to check fusidic acid binding on 70S/50S-EF-G-[3H]GDP complex a. with 70S and b. with 50S ribosome subunits from *E. coli*. Fusidic acid was incubated with 70S/50S, EF-G and [3H]GTP for complex formation and the complex was filtered using nitrocellulose filter. The stable complexes retained on the filter was monitored by [3H]GDP counts (CPM plotted on Y-axis). X-axis indicates the complexes formed with wild type (WT) or different mutant EF-Gs from *S. aureus* with or without fusidic acid. To check the background count [3H]GTP alone was filtered. As a negative control for the complex formation *S. aureus* wild type (WT) EF-G, [3H]GTP and 70S/50S was incubated without addition of any fusidic acid (WT-FA). Y- axis indicates the different scintillation counts obtained for different reactions. Abbreviations for amino acids: F, Phe; L, Leu; M, Met; I, Ile; G, Gly; V, Val; P, Pro

**Tripeptide formation by different EF-G mutants from *Staphylococcus aureus***

The activity of the EF-Gs was tested in a tripeptide formation assay. First, a purified initiation complex programmed with an mRNA encoding fMet-Leu-Ile-top was incubated with an elongation mix containing components for Met-Leu dipeptide formation. Further, this dipeptide complex was incubated with EF-G for translocation together with other components for Met-Leu-Ile tripeptide formation. The extent of tripeptide formation was monitored by High performance liquid chromatography (HPLC) (Fig. 4). Since the rate of peptide bond formation is much faster than the rate of translocation, the rate of tripeptide formation was limited by the translocation activity of EF-G.

**Figure 4.** Translocation assay with WT *E. coli*. Region 1-ribosomes with only fMet tRNA, Region 2- ML dipeptide, Region 3- MLI tripeptide formed after EF-G mediated translocation.
For the tripeptide formation assay one primary fusidic acid resistant mutant of *S. aureus* with the single mutation Phe88Leu, and two other mutants with additional fitness compensatory mutations Phe88Leu+Met16Ile and Phe88Leu+Met16Val were used (Nagaev et al., 2001). The activity of these mutants in translocation was checked in comparison with the activity of the wild type EF-Gs from *S. aureus* and *E. coli* (Fig. 5). The primary mutant Phe88Leu showed very little tripeptide formation, whereas the fitness compensatory mutant Phe88Leu+Met16Ile and Phe88Leu+Met16Val showed 50% tripeptide formation compared to the wild type (Fig. 5).

![Figure 5. Tripeptide formation assay with wild type and mutant EF-Gs](image)

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**Figure 5. Tripeptide formation assay with wild type and mutant EF-Gs** Phe88Lue, Phe88Leu+Met16Ile and Phe88Leu+Met16Val from *S. aureus*. The pmol of tripeptide formed were analysed in HPLC as shown in fig 4. Abbreviations for amino acids: F, Phe; L, Leu; M, Met; I, Ile; G, Gly; V, Val;

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**Exchange of guanine nucleotide diphosphate (GDP) from free and ribosome bound EF-G**

The rates of GDP exchange for the *S. aureus* EF-G mutants Phe88Lue, Phe88Leu+Met16Val, Phe88Lue+Met16Ile were estimated using a fluorescent derivative of GDP (mant-GDP) in a stopped-flow apparatus equipped with fluorescence detector. EF-G (wild-type or mutant) was first bound to mant-GDP, which resulted in enhanced fluorescence from mant-GDP. This EF-G-mant-GDP complex was rapidly mixed with excess amount of unlabelled GDP, followed by the recording of time dependent fluorescence decrease due to the exchange of mant-GDP with unlabeled GDP (nonfluorescent) (Fig. 6) (Gao et al., 2007). The same experiments were repeated in the presence of 70S ribosome to measure the GDP exchange rate in the presence of ribosome (Appendix B). The rate of decrease of the mant fluorescence was calculated,
which reflected the rate constant ($K_{\text{off}}$) for GDP to the EF-Gs. The results ($K_{\text{off}}$ value) are summarized in figure 7. The results indicate that the dissociation rate of mant–GDP from free EF-Gs was extremely rapid with $K_{\text{off}} = 146 – 230 \text{ s}^{-1}$, but in the presence of the ribosome the rate of GDP exchange was significantly reduced with $K_{\text{off}} = 66-119 \text{ s}^{-1}$ (Fig. 7, Table 2 and Appendix B). It is noteworthy that in the presence of the ribosome all the mutant EF-Gs showed somewhat slower rate of GDP exchange compared to the WT, whereas in the free state the single mutant Phe88Leu showed $K_{\text{off}}$ similar to that of the WT (Fig 7).

![Figure 6. Dissociation of mant-GDP from S. aureus WT EF-G, induced by rapid mixing with excess amounts of unlabeled GDP as monitored by the decrease of the florescence in stopped-flow apparatus. R.F.I-Rate of florescence intensity](image1)

![Figure 7. Dissociation of mant-GDP from free and ribosome–bound EF-G from WT and mutant S. aureus Phe88Leu, Phe88Leu+Met16Ile and Phe88Leu+Met16Val. The dissociation of mant-GDP from ribosome bound](image2)
EF-G (magenta) or from free EF-G (blue) was induced by rapid mixing with unlabeled GDP and in excess was monitored by the decrease of mant-fluorescence in the stop flow apparatus. Abbreviations for amino acids: F, Phe; L, Leu; M, Met; I, Ile; G, Gly; V, Val;

Over expression of mutant EF-Gs from Staphylococcus aureus

To compare the results obtained with the Phe88Leu group of fusidic acid resistant mutants it is necessary to carry out similar experiments with other fusidic acid resistant mutants. Genes encoding such non-isogenic mutant EF-Gs from S. aureus Ala71Val + Asp189Gly + Pro406Leu, Arg464Ser and Ala376Val + Leu461Phe + Asp463Gly + Ala655Ser were previously cloned and mutant proteins now over-expressed and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 8). The results of the SDS-PAGE showed that all the above mentioned EF-G proteins over-expressed clearly (Fig. 8).

![Figure 8](image)

**Figure 8.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of over-expressed EF-G proteins from *S. aureus* Ala71Val + Asp189Gly + Pro406Leu, Arg464Ser and Ala376Val + Leu461Phe + Asp463Gly + Ala655Ser The arrow indicates the position of EF-G on the gel.

Since these three mutants over expressed in small scale it is planned in the future to grow them in large scale and thereby to purify them using Ni^{2+} column chromatography technique.
Discussion

Can fusidic acid bind to mutant EF-Gs from *S. aureus*?

In order to analyse the mechanism of fusidic acid resistance in *S. aureus* and the fitness cost associated with it, six *fusA* mutants were tested. One of the main aims of this study was to link the fitness cost with the fusidic acid resistance, therefore mutants were selected based on whether they had primary mutations or fitness compensatory mutations. Data obtained from the nitrocellulose filter-binding assay indicated that all the mutant EF-Gs studied were more or less equally impaired in fusidic acid binding, even though the mutations are located at different domains on EF-G (Figure 9). According to Hansson et al., 2005 there are three distinct clusters of mutations mapping to the domain I (green in Fig. 9), domain III (pink in Fig. 9) and domain V (blue in Fig. 9) and it is noteworthy to see that fusidic acid resistant mutations are clustered in the interface of a pocket formed by these three domains.

![Figure 9](image)

**Figure 9**: Location of the fusidic acid resistance EF-G mutants (Table 1) and the switched II region (yellow) on the EF-G structure (Original figure adopted from PDB code IFNM).

Fusidic acid resistance in Phe88Leu from structural viewpoint

The first three mutants selected for this study have the primary Phe88Leu mutation that causes fusidic acid resistance. Phe88Leu has been identified previously as one of the major fusidic acid resistant mutations in other bacteria as well, such as *Thermus thermophilus* and
Further, the alignment of the relevant regions of the EF-G from *S. aureus* (Sa) and *T. thermophilus* (Tt) also indicated that this Phe residue is conserved in these two species and is very closely placed with well-characterised Thr84Ala mutation in *T. thermophilus* (Fig. 10)

| Tt 83 | DTPGHVDFTYVERSMRVLGAIVVFDSSQGVEPQSETVWRQAEKYHVPRIAFANMKDTGADLMVIRTMQER |
| Sa 81 | DTPGHVDFTYVERSLRVLGAVTVLDAQSGVEQETVWRQATTYGVPRIVFVNKDLGNFEYSVSTLHDR |

Figure 10. Alignment of the relevant regions of EF-G from *S. aureus* (Sa), *T. thermophilus* (Tt)

Previous biochemical and structural studies of the *Thermus thermophilus* fusidic acid resistant mutant Thr84Ala indicates that Thr84 is located in the DXXG consensus motif (DTPG sequence in many EF-Gs) in the switch II region of EF-G, which is common to all G-proteins. This motif is involved in the conformational change on the GTP and GDP state of the G-proteins; therefore mutations in this region may affect the conformational changes needed for (i) GTP/GDP binding and exchange on EF-G, as well as (ii) EF-G mediated GTP hydrolysis important for EF-G function in translocation. The mutations impaired in GTP hydrolysis are naturally resistant to fusidic acid since fusidic acid traps EF-G on the ribosome only in the GDP state. In addition to that, previous studies showed that the Thr84Ala mutation in *T. thermophilus* EF-G leads to the loss of function of EF-G in addition to the fusidic acid resistance (Martemyanov et al., 2001; Nagaev et al., 2001).

The ribbon model of the tertiary structure of *T. thermophilus* EF-G, and its amino acid sequence, indicate that Phe90 (Phe88 in *S. aureus*) is located close to Thr84Ala conferring fusidic acid resistance in *T. thermophilus*. Phe90 is a part of the small hydrophobic core that stabilizes the conformation of the tip of the switch II region (Hansson et al., 2005). In the fusidic acid resistant mutation Thr84Ala (*T. thermophilus*), the Phe side chain appears to be blocking the domain interfaces (Fig. 12, magenta trace), which confers fusidic acid resistance. On the other hand, in the fusidic acid hypersensitive mutation Gly16Val in *T. thermophilus* the side chain is away from the domain interfaces (Fig. 12, yellow trace) keeping it open for fusidic acid binding. The role of this Phe residue in EF-G has been highlighted as a regulator that act as a gatekeeper promoting the binding of fusidic acid (Fig. 12, green trace). This indicates the importance of Phe88 (Phe90 in *T. thermophilus*) in fusidic acid resistance. In *S. aureus* when Phe88 mutated to Leu, the Leu side chain may stick out in the domain interface leading to fusidic acid resistance.
Fig-12. The different conformations of switch II, in particular Phe90, in EF-G mutants Thr84Asp- magenta (fusidic acid resistant mutant), Gly16Val-yellow (fusidic acid sensitive mutant) and wild type -green in *T. thermophilus*, (Hansson et al., 2005). Phe90 in *T. thermophilus* is equivalent to Phe88 in *S. aureus*.

**Phe88Leu strain shows growth impairment due to inactivity in tRNA translocation**

In the tripeptide assay when activity of Phe88Leu was checked for its tRNA translocation compared to wild type EF-G, the primary mutant Phe88Leu showed very poor translocation. This indicates that the Phe88Leu mutation inactivates the EF-G, leading to poor growth of the strain.

**Fitness compensation by secondary mutations in Met16**

Results of the tripeptide assay further indicated that compared to EF-G from wild type *S. aureus*, the fitness compensatory mutants Phe88Leu +Met16Val and Phe88Leu +Met16Ise showed better tRNA translocation (around 50%). This gained ability in tRNA translocation may be due to the secondary mutations at the Met16 residue, resulting the function of their EF-Gs.

**What causes the fitness compensatory mutants to be better in translocation?**

The improvement in translocation of the fitness compensatory mutations Phe88Leu +Met16Val and Phe88Leu +Met16Ise can be due to the improvement in i) GTP/GDP exchange ii) better binding of EF-G to ribosome or iii) due to other reasons affecting the function of EF-G.

The GTP/GDP exchange assay results indicated that there is not much difference in GTP/GDP exchange rate among the primary mutant and the fitness compensatory mutants Phe88Leu +Met16Val and Phe88Leu +Met16Ise in the free state as well as when the EF-G were bound to ribosome. Furthermore, there was not much difference between wild type *S. aureus* and these three mutants (Phe88Leu +Met16Val and Phe88Leu +Met16Ise) in GTP/GDP exchange rate when EF-G s were bound to ribosome. Similar results were obtained
by Wilden et al., (2006), indicating that nucleotide binding to EF-G stabilizes the EF-G better when the EF-G is bound to a ribosome.

Further with my filter binding results it was indicated that there was not much difference among these three mutant EF-Gs in binding to the ribosome. Therefore the gained ability in tRNA translocation in the secondary mutations at Met16 residue may not be due to the differences by the EF-G binding to the ribosome or due to the difference in the GTP/GDP exchange rate but can be due to some other fact improving the function of the EF-G. Whether this mutation affects EF-G function by interfering with the conformational change on EF-G remained an open question.
MATERIALS AND METHODS

Table 1. Strains of *S. aureus* (adopted from Nagaev et al. 2001)

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1 MIC, minimum inhibitory concentration. Susceptibilities of *S. aureus* strains to fusidic acid were analysed using MIC values by using the E test strips.

2 CI, competitive index - this shows the relative fitness value of each strain and is calculated as the ratio of mutant to wild type bacteria.

3 FCM, Fitness compensatory mutant

4 underlined A71V and A376 are putative fitness-compensating mutations

E. coli strain BL21(DE3) competent cells and PET 30 plasmid were used in the transformation experiment.

Chemicals and Buffers

ATP and GTP were purchased from Amersham Biosciences. Fusidic acid was purchased from Leopharma and [³H] GDP from GE Healthcare. Nitrocellulose Protran BA 85 filters were from Whatman GMBH, Germany. Scintillation liquid (Quicksafe flow 2) used for the tripeptide assay was from Zinsser analytic, GB. Scintillation liquid (Filtersafe) used for the filter-binding assay was from Packard Biosciences BV, Netherland. mRNA encoding Met-Leu-Iso,tRNA^[Met], bulk tRNA, tRNA^[Ile], tRNA^[Leu], EF-Tu, EF-Ts, Ribosomes of *E. coli* MRE600 strain, tRNA synthetases, Ile-tRNA synthetase and Leu-tRNA synthetase were available in the laboratory. *S. aureus* wild type and different EF-G mutants were a kind gift from Chenhui Huang.

Polymix buffer contained (final concentrations of the components) 5 mM ammonium chloride, 95 mM potassium chloride, 0.5 mM calcium chloride, 8 mM putrescine, 1 mM spermidine 5 mM potassium phosphate and 1 mM 1,4-dithioerythritol (Jelenc et al., 1979). In case of
Hepexpolymix buffer, 10 mM Hepex was added instead of 5 mM potassium phosphate in the polymix buffer and all the other components were the same as polymix buffer.

**Sodium dodecyl sulphate–polyacrylamide gel electrophorosis (SDS PAGE)**

30% acrylamide gels were cast in two steps, first the separating gel and then the stacking gel. 10 ml of separating gel contained 3.3 ml of sterile water, 4 ml of 30% w/v acrylamide:N-N’-methylene-bis-acrylamide mix, 2.5 ml of 1.5 M Tris (pH 8.8 adjusted with HCl), 0.1 ml of 10% SDS, 0.1 ml 10% ammonium persulfate and 0.004 ml of TEMED. This was poured into the glass plate sandwich and allowed solidify (30 min). Then 5 ml of stacking gel was prepared containing 1.1 ml of sterile water, 2.5 ml of 30% w/v acrylamide:N-N’-methylene-bis-acrylamide mix, 1.3 ml of 1.5 M Tris (pH 8.8 adjusted with HCl), 0.05 ml of 10% SDS, 0.05 ml 10% ammonium persulfate and 0.002 ml of TEMED. This was added on top of the solidified separating gel and a comb inserted before allowing the gel to get solidified (30 min). Then the comb was removed and the glass sandwich fixed inside a container filled with running buffer (pH 2.3 adjusted with HCl) containing 45 g Tris, 216 g glycine, 15 g SDS per 3 L distilled water. Samples were prepared by adding 15 µl of protein and 5 µl of 3 X SDS PAGE loading dye (2.9 ml 1M Tris (pH 6.8 adjusted with HCl), 3 ml 20% SDS, 3 ml 100% glycerol, 0.006 g bromophenol blue) and incubating for 5 min at 37 °C. They were then vortexed gently and 20 µl of sample was loaded per well. After electrophoresis the gel was stained with Coomassie blue staining dye (90 ml methanol: H₂O 1:1, 10 ml 100 % glacial acetic acid, 0.25 g coommasie blue per 100 ml) and kept on a shaker. After 4 hours discard the staining dye and added de-staining solution (12 % v/v isopropyl alcohol and 5 % acetic acid) to cover the gel and kept on a shaker until the gel got cleared to see the locations of the proteins.

**Transformation of EF-G mutant proteins**

Kanamycin resistant PET 30 plasmid cells (2 µl) (from Norvagen) having respective EF-G mutant genes Phe88Leu (316), Phe88Leu+Met16Val (502), Phe88Lue+Met16Ile (497) were transformed into *E. coli* BL21(DE3) competent cells (from Invitrogen) (15 µl) and kept on ice for 45 min. After giving a heat shock for 1 minute at 42°C, the cells were cooled on ice (5 minutes) and then grown in Luria broth (LB, per L 10 g NaCl, 5 g yeast extract, 10 g tryptone, 2.9 ml of 1M CaCl₂, 9.5 ml of 20% glucose, 200 µl per tube) for 1 hour and plated (200 µl/plate) on Luria agar with kanamycin (LA+Kan, 20 g agar and 50 mg kanamycin per L LB) and kept overnight at 37 °C oven.

**Filter binding assay for EF-Gs FA binding**

Reaction mixes were made with 1 µM ribosome (70S or 50S), 4 µM EF-G (WT *S. aureus* or its mutants), 3 mM fusidic acid and 2 µM [³H] GDP in complex formation buffer (CFB) having, 10 mM Tris (pH 7.5 adjusted with HCl) 10 mM NaH₄Cl, 10 mM Mg- acetate, 1 mM DTT (1,4- dithioerythritol) and incubated on ice for 1 hour. 20 µl of the sample was filtered through pre-soaked nitrocellulose filters (0.45 µM, Protran BA 85) in CFB containing 0.3 mM fusidic acid, followed by a washing step with 5 ml of ice cold CFB containing 0.3 mM fusidic acid. After that the filters were soaked in scintillation liquid for 30 minutes with vigorous shaking, and the scintillation was measured in a Beckman LS6500 multipurpose scintillation counter.
**Translocation Kinetics**

Two mixes A and B were prepared in polymix buffer. Mixture A: 1 μM *E. coli* 70S ribosomes, 4 μM initiation factor 1 (IF1), 4 μM initiation factor 3 (IF3), 1 μM mRNA (Met-Leu-Ile) and 1 mM GDP was first incubated at 37°C for 5 minutes. Then 2 μM [³H]fMet-tRNA and 4 μM initiation factor 2 (IF2) was added. Mixture B was made with 10 μM EF-Tu, 5 μM EF-Ts, 280 μM total tRNA (t-Bulk), 500 mM of each of the amino acids Leu and Ile, 0.5 μM of each of Leu and Ile synthetases, 5 μM EF-G (WT and different mutants), 1 mM of ATP and 1 mM of GTP. The two mixes were separately incubated for 5 - 10 min at 37°C and then mixed together for 1 min; the reaction was quenched by adding formic acid to the final concentration of 15%. The quenched reaction mix was centrifuged at 30 x 3.75 x g for 15 min, and the pellet was dissolved in 170 μl of 0.5 M KOH, to release the peptides by cleaving the RNAs. 10-15 μl of 100% formic acid was added to precipitate all the RNAs in the solution. The sample was centrifuged twice at 30 x 3.75 x g for 15 min each. Then the supernatant was loaded on a C-18 column (Merck), equilibrated and eluted with 42% methanol and 0.1% trifluoroacetic acid in water by HPLC (High performance liquid chromatography) (Waters™ 717) to check the extent of tripeptide (MLI) formation.

**GDP exchange on Mutant EF-G’s**

To study the GDP exchange on mutant and WT *Staphylococcus aureus* EF-G, 4 μM of EF-G was incubated with 2 μM of mant-GDP, a fluorescent derivative of GDP, at 37°C for 10 minutes in a Hepes-polymix buffer. Then this complex was mixed with 200 μM of unlabelled GDP. The rate of GDP exchange was monitored by measuring the decrease of mant-GDP fluorescence in a Sx20 stopped-flow apparatus (Applied Photophysics). Stopped flow instrument is a rapid mixing device used to study the chemical kinetics of reaction in a solution. When fluorescence labelled mant GDP was mixed with unlabeled GDP, the level of fluorescence in the reaction solution gets reduced. This florescence reduction is measured using the fluorescence spectrophotometer coupled to the stopped flow instrument.

To study the GDP exchange in the presence of ribosomes, 1 μM EF-G was incubated with 1 μM 70S ribosomes in the presence of mant-GDP before mixing with 400 μM of unlabeled GDP.

**Overexpression of EF-G proteins**

Single colonies were picked and inoculated into LB+kanamycin (1 μg/1 ml) medium. When the OD600 value reached 0.5, isopropyl-beta-D-thiogalactopyranoside (IPTG), dioxane grade, (1 μg/1 ml) was added to the cultures and kept three hours for the overexpression of the EF-G proteins. The cells were pelleted by centrifuging at 2000 x g for 20 minutes followed by washing the pellet with Tris buffer (pH 7.5 adjusted with HCl and Tris 20 mM, NaCl 100 mM) and centrifuging again for 20 minutes at 2000 x g. Finally the pelleted cells were stored at -80 °C. Then 500 μl of cell paste was resuspend in TMN 200 buffer (40 mM Tris, 10 mM MgCl₂, 200 mM NaCl) and the over- expression of the EF-G protein analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophorosis (SDS PAGE).
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Appendix B. Dissociation of mant-labelled nucleotides from free and ribosome bound EF-G’s of *S. aureus* WT and its mutants, F88L (316), F88L+ M16V (502) & F88L+ M16I (497). The dissociation of mant-GDP from freeEF-G or from ribosome-bound EF-G was induced by, rapid mixing with excess amounts of unlabeled GDP and monitoring the fluorescence on Stop flow apparatus.