ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1991, p. 2366–2370
0066-4804/91/112366-05$02.00/0

Vol. 35, No. 11

Comparison of the Tu Elongation Factors from *Staphylococcus aureus* and *Escherichia coli*: Possible Basis for Elfamycin Insensitivity

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Received 10 June 1991/Accepted 27 August 1991

In a previous study (C. C. Hall, J. D. Watkins, and N. H. Georgopapadakou, Antimicrob. Agents Chemother. 33:322–325, 1989), the elongation factor Tu (EF-Tu) from *Staphylococcus aureus* was found to be insensitive to a series of kirromycin analogs which were inhibitory to the EF-Tu from *Escherichia coli*. In the present study, the EF-Tu from *S. aureus* was partially purified and characterized. Its apparent molecular mass was approximately 41,000 Da, and the enzyme copurified with EF-Ts (molecular mass, 34,000 Da). *S. aureus* EF-Tu differed from its *E. coli* counterpart in that it bound negligible amounts of [^{3}H]GDP, in addition to being insensitive to pulvomycin and aurodox (50% inhibitory concentrations, approximately 100 and 1,000 μM, respectively, versus 2 and 0.2 μM, respectively, for *E. coli*). The results are consistent with the formation of a stable EF-Tu · EF-Ts complex that affects the interaction of EF-Tu with guanine nucleotides and inhibitors.

Elongation factor Tu (EF-Tu) is a major cytoplasmic protein involved in the elongation stage of protein biosynthesis in bacteria (14). It catalyzes the binding of aminoacyl-tRNA to the ribosome, a process which requires the prior formation of an aminoacyl-tRNA · EF-Tu · GTP complex. The formation of this ternary complex is mediated by elongation factor Ts (EF-Ts), which converts EF-Tu to a GTP-binding conformation. EF-Tu has been purified from a variety of bacteria, including archaebacteria (10, 22), and its molecular mass ranges from 43,000 Da for the enzyme from *Escherichia coli* (12, 13) to 52,000 Da for the enzyme from *Thermus thermophilus* (1). The enzyme from *E. coli* has been characterized extensively (8, 9, 14). Inhibitors of *E. coli* EF-Tu include kirromycin and its analogs (efrotomycin, aurodox) (15) and the structurally unrelated inhibitor pulvomycin (21). A key feature of both types of inhibitors is the induction of a GTP-binding conformation of EF-Tu.

Staphylococcus aureus is a gram-positive pathogen of increasing concern because of its widespread incidence and frequent resistance to antibiotics (2, 11, 20). In our previous studies with EF-Tu from this organism, we compared the inhibitory activity of kirromycin analogs in poly(U)-dependent poly(Phe) synthesis assays derived from *S. aureus* and *E. coli* (6). In contrast to *E. coli* EF-Tu, in the *S. aureus* system all kirromycin analogs tested had 50% inhibitory concentrations of 1,000 μM or greater, indicating an intrinsic insensitivity of *S. aureus* EF-Tu to these agents.

In this study, we examined the interactions of *S. aureus* EF-Tu with its ligands GDP, GTP, and EF-Ts. These interactions differed from those of the *E. coli* factor and suggest a possible basis for the kirromycin insensitivity of *S. aureus*.

MATERIALS AND METHODS

Organisms and growth conditions. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were obtained from the American Type Culture Collection (Rockville, Md.). Both organisms were grown in Luria broth at 37°C to late-log phase and collected as cell pastes.

Antibiotics. Aurodox was obtained from Roche Laboratories (Nutley, N.J.), and efrotomycin was obtained from Merck Sharp & Dohme Research Laboratories (Rahway, N.J.). Pulvomycin was a generous gift of William Higgins, Merrell Dow Research Institute (Strasbourg, France).

Other materials. [^{3}H]GDP (specific activity, 8.3 Ci/mmol) and [γ-^{32}P]GTP (specific activity, 20 to 40 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, Mass.). Phenylmethylsulfonyl fluoride (PMSF) and molecular mass standards (bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, and trypsin inhibitor) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Filtron X scintillation fluid was purchased from National Diagnostics (Somerville, N.J.). Other reagent sources have been described previously (6).

***S. aureus* EF-Tu purification.** EF-Tu was purified from *S. aureus* ATCC 25923 by using an adaptation of an earlier method (10) and by using the lysis procedure of Georgopapadakou et al. (4). A 200-g sample of cell paste was resuspended in 200 ml of 50 mM Tris-HCl (pH 7.5) containing 0.1 M KCl, 1.1 mM PMSF, and 0.05 mg of trypsin inhibitor per ml. A total of 8.0 ml of 1.5 mg of lysostaphin per ml and 3 mg of lysozyme per ml in 50 mM Tris-HCl (pH 7.5) was added, and the mixture was incubated for 40 min at 25°C. Then, 6.6 ml of 4% sodium deoxycholate and 1.3 ml of 1 mg of DNase I per ml in 50 mM Tris-HCl (pH 7.5) and 8.0 ml of 0.64 M $MgCl_2$ containing 32 mM dithiothreitol (DTT) were added, and the mixture was incubated for an additional 15 min at 25°C. The cell debris and ribosomes were removed by two successive centrifugations (36,600 × *g* for 40 min, 140,000 × *g* for 150 min). The final supernatant was fractionated with 40 to 70% ammonium sulfate. The resulting pellet was resuspended in 40 ml of simplified procedure buffer (SP buffer; 64.4 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM PMSF) containing 0.05 mg of trypsin inhibitor per ml and was dialyzed overnight against 4 liters of SP buffer.

The dialysate was diluted with 80 ml of SP buffer and was added to a DEAE–Sepharose CL-6B column (2.5 by 56 cm), which was then eluted at a flow rate of approximately 1 ml/min. The column was washed with 165 ml of SP buffer at

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the same flow rate and was eluted with a gradient of 0 to 0.4 M NaCl in SP buffer (total gradient volume, 1,000 ml). *S. aureus* EF-Tu was monitored throughout purification by its stimulation of poly(Phe) synthesis in an EF-Tu-deficient *E. coli* poly(U)-dependent poly(Phe) synthesis system. The protein compositions of EF-Tu samples during purification were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gels containing 10% (wt/vol) acrylamide and 0.27% *N,N'*-methylene-bisacrylamide.

Pooled EF-Tu fractions (90 ml, 0.7 mg/ml) from the ion-exchange column were concentrated to 13.5 ml by ultrafiltration (Amicon PM-10 membrane), added to a Sephacryl S-200 column (2.5 by 77 cm), and eluted with SP buffer at a flow rate of 15 ml/h.

The pooled EF-Tu fractions (55 ml) from the Sephacryl S-200 column were concentrated to 6.5 ml by ultrafiltration and were applied to a Sephadex G-100 column (2.5 by 32 cm) equilibrated with SP buffer containing 0.1 mM GDP. The column was eluted with SP buffer at a flow rate of 0.26 ml/min.

***E. coli* EF-Tu purification.** EF-Tu was purified from *E. coli* ATCC 25922 by the method of Leberman et al. (10).

Poly(Phe) assay. The *E. coli* poly(Phe) synthesis assay was performed as described previously (6).

For the *E. coli* EF-Tu-deficient poly(Phe) synthesis assay of *S. aureus* EF-Tu, *E. coli* ribosomes (16) were used with *E. coli* EF-G purified by the method of Leberman et al. (10). The 200-μl assay mixture contained the following: 50 mM Tris-HCl (pH 7.6), 80 mM NH_4Cl, 80 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 0.153 optical density units of *E. coli* ribosomes at 260 nm (OD_{260} units), 65 pmol of [^{3}H]Phe-tRNA, 2.5 μg of *E. coli* EF-G, 200 nmol of GTP, 175 μg of poly(U), and 25 μl of the *S. aureus* EF-Tu fraction to be tested. The reaction was started by the addition of GTP and poly(U), and after a 5-min incubation at 37°C, the reaction was terminated by adding 5 ml of 5% trichloroacetic acid. The mixture was heated for 5 min at 95°C, cooled to room temperature, and filtered on nitrocellulose filters (pore size, 0.045 μm). The filters were washed twice with 5 ml of 10% trichloroacetic acid and were counted in 10 ml of Filtron X.

The *S. aureus* poly(Phe) synthesis assay was performed as described by Hall et al. (6).

GDP binding assay. *E. coli* or *S. aureus* EF-Tu was assayed by the [^{3}H]GDP binding method of Miller and Weissbach (12). Incubations with 2.5 μM [^{3}H]GDP (specific activity, 0.083 Ci/mmol) were performed for 5 min at 37°C, rather than at 0°C, to increase the dissociation of the GDP that was already bound.

GTPase assay. EF-Tu-dependent hydrolysis of [γ-^{32}P]GTP to [^{32}P]PO_4 was determined by the molybdate extraction procedure of Sander et al. (18).

RESULTS

***S. aureus* EF-Tu preparation.** Following breakage of *S. aureus* cells, ribosomes were removed by centrifugation and the supernatant was fractionated with ammonium sulfate. Although ammonium sulfate fractionation did not result in net purification (Table 1), it nevertheless proved necessary for removal of high-molecular-mass proteins which were difficult to remove at subsequent purification steps. EF-Tu was subsequently isolated by a combination of ion-exchange chromatography and gel-exclusion chromatography.

As shown in Fig. 1A, EF-Tu activity emerged from the DEAE-Sepharose CL-6B column as a complex peak. The leading edge of the peak (fractions 44 to 51 in Fig. 1A) contained the EF-Tu · EF-Ts complex, while the later fractions (fractions 52 to 56) contained EF-G [as assayed by an EF-G-deficient poly(Phe) synthesis assay]. The EF-G fractions showed a low-level, variable binding of [^{3}H]GDP which exceeded the binding observed for the EF-Tu fractions. This is in contrast to *E. coli*, in which the major [^{3}H]GDP-binding peak contains the EF-Tu fractions (12, 13).

The fractions of the DEAE-Sepharose CL-6B column which contained the EF-Tu · EF-Ts complex (fractions 44 to 51 of Fig. 1A) were added to the Sephacryl S-200 column, and the elution profile is shown in Fig. 1B. In this step, the

TABLE 1. Purification of *S. aureus* EF-Tu

Purification step	Total protein (mg)	Sp act [pmol of poly(Phe)/mg/min]	Purification (fold)
Cell breakage	2,025	4.2	1
40 to 70% $(NH_4)_2SO_4$	845	2.0	0.5
DEAE-Sepharose CL-6B	18.5	174	41
Sephacryl S-200	1.9	1,160[a]	276

[a] Specific activity of pure *E. coli* EF-Tu, 4,260 pmol/mg/min.

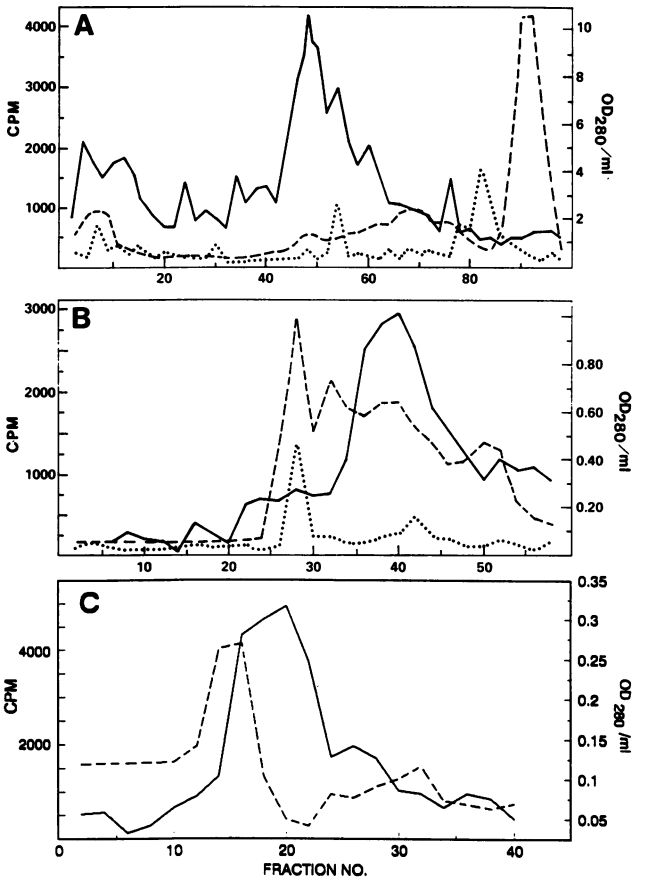

FIG. 1. Column elution profiles during *S. aureus* EF-Tu purification. (A) DEAE-Sepharose CL-6B chromatography (10-ml fractions); (B) Sephacryl S-200 chromatography (5-ml fractions); (C) Sephadex G-100 chromatography (5.2-ml fractions). Assays were performed on even-numbered fractions. ——, counts per minute of poly(Phe) formed; · · ·, counts per minute of [^{3}H]GDP formed; – – –, OD_{280}/ml.

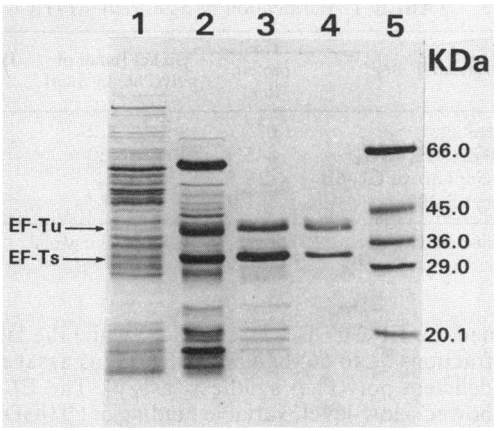

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of active fractions of *S. aureus* EF-Tu at different stages of purification. Lanes: 1, 40 to 70% ammonium sulfate fraction; 2, DEAE–Sepharose CL-6B; 3, Sephacryl S-200; 4, Sephadex G-100; 5, molecular mass standards.

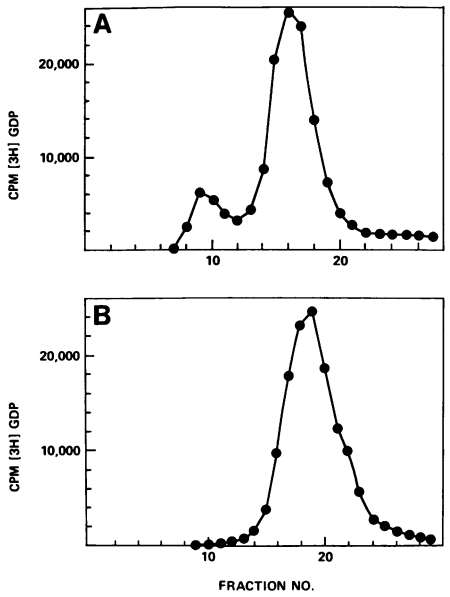

FIG. 3. Binding of [^{3}H]GDP by *E. coli* EF-Tu (A) and *S. aureus* EF-Tu (B) assayed by gel filtration. A 48-μg sample of *E. coli* or *S. aureus* EF-Tu was incubated in 400 μl of 2.5 μM [^{3}H]GDP (59 cpm/pmol) in SP buffer, applied to a Sephadex G-25 column (1 by 13 cm), and eluted with SP buffer (0.5-ml fractions).

S. aureus EF-Tu · EF-Ts complex was separated from other proteins (Fig. 2). The [^{3}H]GDP-binding activity recovered from this column was low and erratic. *S. aureus* EF-Tu preparations consistently contained substantial amounts of EF-Ts, and this complex was much more difficult to separate into components than the EF-Tu · EF-Ts complex of *E. coli* was. *S. aureus* EF-Tu persisted as an EF-Tu · EF-Ts complex even after passage through a Sephadex G-100 column in the presence of 0.1 mM GDP (Fig. 1C and 2), conditions which facilitate the dissociation of *E. coli* EF-Tu · EF-Ts. The *S. aureus* EF-Tu activity that eluted from the Sephadex G-100 column appeared as a single peak at fraction 20 (Fig. 1C), with an elution volume distinct from that of a 45,000-Da protein (ovalbumin), which eluted at fraction 25 under identical conditions. The *S. aureus* EF-Tu activity therefore migrates at a molecular mass substantially higher than the free 41,000-Da subunit of *S. aureus* EF-Tu.

The protein profile after each purification step is shown in Fig. 2, and the enrichment of EF-Tu activity during purification is given in Table 1. The purification in the final Sephadex G-100 column was typically about twofold. A mixture of approximately 1:1 EF-Tu and EF-Ts (molecular masses, 41,000 and 34,000 Da, respectively) was obtained. Small amounts of the isolated 34,000-Da species were recovered from Sephadex G-100 columns, and these exhibited EF-Ts activity, as measured by the ability of the 34,000-Da species to stimulate the exchange of [^{3}H]GDP for tightly bound GDP in purified *E. coli* EF-Tu fractions.

GDP binding of *S. aureus* EF-Tu. Unlike *E. coli* EF-Tu (7), the EF-Tu present in crude *S. aureus* ribosomal supernatant fractions did not bind significantly to GDP-agarose and could not be purified by affinity chromatography (5). Accordingly, at all stages of purification *S. aureus* EF-Tu did not bind [^{3}H]GDP in a nitrocellulose filter assay (12), up to 1 mM [^{3}H]GDP. Since this frequently used EF-Tu assay was not appropriate for this factor, the *S. aureus* EF-Tu was monitored during purification by using an *E. coli* poly(U)-dependent poly(Phe) synthesis system which was deficient in EF-Tu. The *S. aureus* EF-Tu has been shown previously to interact in a functional manner with *E. coli* ribosomes (6).

The apparent failure of purified *S. aureus* EF-Tu to bind [^{3}H]GDP in the nitrocellulose filter assay could result from possible poor retention of the *S. aureus* EF-Tu by nitrocellulose filters. However, no binding of [^{3}H]GDP to *S. aureus* EF-Tu was detected after passage of a sample of EF-Tu containing 2.5 μM [^{3}H]GDP through a Sephadex G-25 column, while *E. coli* EF-Tu showed marked binding of the guanine nucleotide (Fig. 3). Fractions 14 to 20 contained unbound [^{3}H]GDP, and fractions 7 to 12 contained the EF-Tu · [^{3}H]GDP complex. No significant binding of [^{3}H]GDP to the *S. aureus* factor was observed by this method, even when the concentration of [^{3}H]GDP was raised to 1 mM. Accordingly, no spectral changes were observed during dialysis of the *S. aureus* factor against Mg^{2+}-free buffer (Fig. 4). These dialysis conditions have been shown previously to produce a measurable decrease in the OD_{260} of *E. coli* preparations, which corresponds to the loss of bound GDP (12). The absence of such spectral changes with the *S. aureus* factor indicated that there was no tightly bound GDP in the isolated factor. Hence, the *S. aureus* factor neither bound significant amounts of [^{3}H]GDP nor had tightly associated GDP when it was isolated.

GTPase activity of *S. aureus* EF-Tu. Like its *E. coli* counterpart, purified *S. aureus* EF-Tu showed GTPase activity under conditions of enzymatic binding of [^{3}H]Phe-tRNA to ribosomes (Table 2). However, the GTPase activity of purified *S. aureus* EF-Tu was unresponsive to 1 mM aurodox or efrotomycin, in contrast to the *E. coli* factor (Table 3).

In vitro activity of pulvomycin in *S. aureus*. Pulvomycin, which produced stimulation of the GTPase activity of *E. coli* EF-Tu, failed to stimulate the GTPase activity of the *S. aureus* factor at 0.1 mM (data not shown). Pulvomycin was subsequently examined in the poly(U)-directed poly(Phe) synthesis assays derived from *E. coli* and *S. aureus*. It showed a 50% inhibitory concentration of approximately 100 μM, the limit of solubility, in the *S. aureus* system, and a

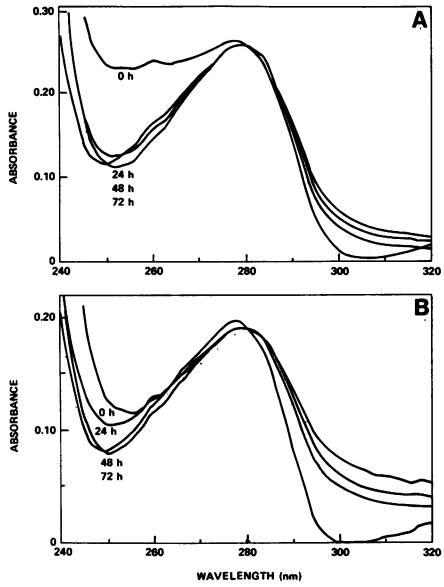

FIG. 4. UV spectra of *E. coli* EF-Tu (A) and *S. aureus* EF-Tu (B) after dialysis. EF-Tu samples were prepared in the absence of added GDP. A 0.4-ml sample of 0.24 mg of *E. coli* EF-Tu or *S. aureus* EF-Tu (from the Sephacryl S-200 column) per ml was dialyzed for the times indicated against 4 liters of 50 mM Tris-HCl (pH 7.8)–1 mM disodium EDTA–5 mM DTT at 4°C. Spectra were normalized to a constant OD_{280} to correct for the amount of protein present.

50% inhibitory concentration of 2 μM in the *E. coli* system (Fig. 5).

DISCUSSION

The partial purification of EF-Tu from *S. aureus* represents the first time that this factor has been isolated from a human pathogen with an EF-Tu naturally resistant to kirromycin. The molecular mass of *S. aureus* EF-Tu is not significantly different from that of the *E. coli* factor. The molecular mass of *S. aureus* EF-Ts, on the other hand, is significantly higher than the 28,000-Da *E. coli* EF-Ts (14). However, molecular masses of EF-Ts as high as 62,000 Da (chloroplast) have been reported previously (3).

An unusually stable association of EF-Tu with EF-Ts is not unique to *S. aureus*; it has been reported previously for *T. thermophilus* (1). In *S. aureus*, however, the tight EF-Tu · EF-Ts association may play a role in the decreased sensitivity of EF-Tu to kirromycin analogs and pulvomycin. Binding of EF-Ts to EF-Tu has been shown to be inhibited by kirromycin analogs in *E. coli* EF-Tu (15). A very stable EF-Tu · EF-Ts complex, which also shows kirromycin insensitivity and decreased guanine nucleotide binding, has been reported for mitochondrial EF-Tu in mammalian cells (19). The EF-Tu of the phylogenetically close *Lactobacillus brevis* is resistant, as is *S. aureus* EF-Tu, to kirromycin analogs and pulvomycin (23).

The uniformly low level of [^{3}H]GDP binding to *S. aureus* EF-Tu, as measured by nitrocellulose assays or molecular sieving columns, is probably due to a rapid GDP dissociation rate. Rapid dissociation of bound GDP from EF-Tu is a likely consequence of a very stable EF-Tu · EF-Ts complex. Greatly reduced binding of [^{3}H]GDP to EF-Tu in nitrocellulose filter binding assays has been observed previously for mitochondrial EF-Tu (17, 19). Thus, a very low dissociation rate for bound GDP may not be essential for EF-Tu function.

Like all characterized EF-Tus, the *S. aureus* factor shows GTPase activity under conditions of enzymatic binding of [^{3}H]Phe-tRNA to *E. coli* ribosomes. This suggests that the isolated *S. aureus* factor interacts in the necessary functional manner with guanine nucleotides. The GTPase activity of purified *S. aureus* EF-Tu is insensitive to kirromycin analogs and pulvomycin. This confirms our earlier findings of a kirromycin-resistant *S. aureus* EF-Tu (6).

In conclusion, *S. aureus* EF-Tu has a molecular mass

TABLE 2. GTPase activities of *E. coli* and *S. aureus* EF-Tus

Addition	GTPase activity (pmol of [γ-^{32}P]GTP hydrolyzed/min)[a]	
	S. aureus EF-Tu	*E. coli* EF-Tu
EF-Tu	<0.1	0.48
Ribosome + poly(U) + Phe-tRNA	0.74	1.62
EF-Tu + ribosome + poly(U) + Phe-tRNA	1.44	3.30

[a] The assay mixture contained, in 75 μl: 60 mM Tris-HCl (pH 7.8), 30 mM KCl, 30 mM NH_4Cl, 10 mM $MgCl_2$, and 2 mM DTT (standard buffer) and 1 mM [γ-^{32}P]GTP (0.12 Ci/mmol). Where indicated, additions were 24.2 pmol of [^{3}H]Phe-tRNA, 70.4 μg of poly(U), 9.6 μg of *S. aureus* EF-Tu or 7.5 μg of *E. coli* EF-Tu, and 0.051 OD_{260} units of *E. coli* ribosomes (1.3 pmol). Note that ribosomes are the limiting reagent in this experiment.

TABLE 3. Effect of aurodox and efrotomycin on *S. aureus* and *E. coli* EF-Tus

Addition	GTPase activity (pmol of [γ-^{32}P]GTP hydrolyzed/min)[a]	
	S. aureus EF-Tu	*E. coli* EF-Tu
None	0.011	0.095
Aurodox (1 mM)	0.014	1.1
Efrotomycin (1 mM)	0.009	1.1

[a] The assay mixture contained (in 75 μl of standard buffer) 2.6 μM [γ-^{32}P]GTP (2.4 Ci/mmol), 9.9 μg of *S. aureus* EF-Tu, or 8 μg of *E. coli* EF-Tu. Incubation was for 20 min at 30°C.

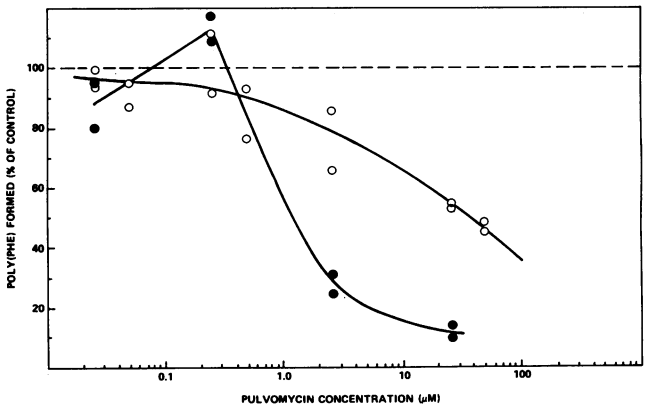

FIG. 5. Effect of pulvomycin on poly(Phe) synthesis in *E. coli* (closed circles) and *S. aureus* (open circles). The total [^{3}H]Phe polymerized in controls was as follows: *S. aureus*, 1 pmol; *E. coli*, 5 pmol.

similar to that of *E. coli* EF-Tu and is capable of interacting functionally with *E. coli* ribosomes. However, it differs substantially from *E. coli* EF-Tu in its interactions with natural ligands (GDP, GTP, EF-Ts) and inhibitors (kirromycin analogs, pulvomycin). The finding that *S. aureus* EF-Tu exists as a tight EF-Tu · EF-Ts complex and is kirromycin resistant may have important implications for the design of antibiotics against this target.

ACKNOWLEDGMENTS

We thank Leo Bowski and Dale Mueller, Fermentation Department, Hoffmann-La Roche, for *S. aureus* cell paste preparations, and Herbert Weissbach, Roche Institute of Molecular Biology, for helpful discussions.

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