Translocation in *Bacillus subtilis*: Characterization of Elongation Factor G by Peptidyl-[\(^{3}\text{H}\)]puromycin Synthesis

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This communication describes the characterization of elongation factor G from *Bacillus subtilis* by the translocation of "native" peptide donors. Translocation was followed by elongation factor G-dependent increase in the synthesis of peptidyl-[\(^{3}\text{H}\)]puromycin using "washed" ribosomes carrying in vivo-bound peptidyl-transfer ribonucleic acid ("native" peptidyl-transfer ribonucleic acid) molecules as peptide donors. Such ribosomes were obtained from cell extracts by washing at a high salt concentration. The use of "native" peptide donors facilitated the study of translocation under conditions that are closer to the in vivo state than those in the methods previously employed.

Elongation of peptides has been studied extensively in *Escherichia coli*, and the protein factors involved have been purified and characterized (10, 12, 15). One of these factors, elongation factor G (EF-G) (9, 11, 17), was shown to be essential for the guanosine 5'-triphosphate (GTP)-dependent translocation of peptidyl-transfer ribonucleic acid (tRNA) molecules from the acceptor site (A site) to the donor site (P site) on the ribosome (24, 25), thus facilitating the binding of the next charged tRNA molecule.

Several methods have been used for measuring the activity of EF-G: (i) complementation of elongation factor T (EF-T) in the synthesis of polyphenylalanine by washed ribosomes and polyuridylic acid (8, 19); (ii) ribosome-dependent hydrolysis of GTP (guanosine triphosphatase [GTPase] activity) (7, 17); and (iii) GTP-dependent translocation of tRNA molecules carrying labeled polypeptides or N-blocked amino acids (5, 6, 20, 26). The charged tRNA molecules are bound to ribosomes in vitro, and their translocation to the P site is measured by the use of puromycin, which has been shown to form peptide bonds only with peptide molecules located at the P site of ribosomes (4, 24).

This paper describes the characterization of EF-G from *Bacillus subtilis* by a new method. In this method translocation was followed by EF-G-dependent increase in the synthesis of peptidyl-[\(^{3}\text{H}\)]puromycin, using "washed" ribosomes carrying in vivo-bound peptidyl-tRNA ("native" peptidyl-tRNA) molecules as peptide donors. Such ribosomes were obtained from cell extracts by washing at a high salt concentration, and their use as peptide donors for the puromycin reaction facilitated the study of translocation under conditions that are closer to the in vivo state than those in the methods previously employed.

### MATERIALS AND METHODS

**Bacteria and growth conditions.** *B. subtilis* 168 was grown in broth containing (per liter): tryptone (Difco), 10 g; yeast extract (Difco), 5 g; sodium chloride, 0.5 g; glucose, 5 to 10 g; and tryptophan, 50 mg. Ten liters of broth was inoculated with 1 liter of culture grown for 16 h in the same medium. The culture was grown in 15-liter fermentors (New Brunswick model FS-314) at 37°C with aeration of 9 liters/min at 400 rpm. Cultures were harvested at the exponential phase of growth at a turbidity of 250 U, as measured in the Klett-Summerson spectrophotometer using filter no. 54. Before the cells were harvested, MgCl₂ was added to a final concentration of 10 mM to prevent cell lysis. The cells were chilled by pouring on an equal volume of ice and sedimented by centrifugation at 15,000 × g for 20 min. The cell pellet was washed twice with cold buffer containing tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.8, 10 mM; magnesium acetate, 10 mM; ammonium chloride, 100 mM; and 6 mM 2-mercaptoethanol or 1 mM dithiothreitol (TAM buffer). In the second washing, 20% glycerol was added to the buffer.

**Preparation of cell extracts and ribosomes.** Cell pellets washed twice with TAM buffer were resuspended in the same buffer (about three times their volume) and sonicated in the Branson sonifier model B-12 (10 s at intensity 4, three times). Deoxyribonuclease (ribonuclease-free, Worthington Biochemicals Corp.) was added to a final concentration of 2 μg/ml. The extracts were then centrifuged for 30
min at 30,000 \times g, and the precipitate was discarded. The clarified extract (S-30) was centrifuged at 105,000 \times g for 3 h to separate the ribosomes from the supernatant (S-100). The ribosomes were washed three times in the same buffer containing 1 M NH₄Cl. The washed ribosomes and the supernatant were stored with 20% glycerol at -20 C.

Ribosome-dependent GTPase activity. Each 0.12-
ml reaction mixture contained Tris-hydrochloride, pH 7.8, 10 mM; ammonium chloride, 100 mM; magnesium acetate, 10 mM; \gamma^{32P}GTP, 1 nmol (250 to 2,500 counts/min per pmol); 1 to 2 absorbance units of ribosomes at 260 nm (A₂₆₀) (washed four times); and protein with EF-G activity, 0.05 to 5 \mu g. Incubation was carried out for 10 min at 37 C. Hydrolyzed GTP was assayed by determining the \textsuperscript{32}P not absorbed by charcoal (8) as follows: 0.1 ml of the reaction was transferred to small polyethylene tubes containing 0.2 ml of 4% charcoal in 0.7 M perchloric acid containing 2.5 mM KH₂PO₄ (16). The tubes were vigorously mixed for 15 s and then centrifuged at 25,000 g for 15 min in a Microfuge model 152. A 0.1-ml portion of the supernatant was counted in 1 ml of Bray solution (3) in a Packard Tri-Carb scintillation counter.

Synthesis of peptideyl-puromycin. Assay of peptideyl-\textsuperscript{3H}puromycin synthesized in vitro was by the method of Pestka (21, 22). Each 0.1-ml reaction contained Tris-hydrochloride, pH 7.8, 10 mM; magnesium acetate, 4 mM; ammonium chloride, 100 mM; GTP (sodium salt), 4 \times 10^{-4} M; ribosomes (washed four times, 5 to 10 A₂₆₀ units); \textsuperscript{3H}puromycin, 100 pmol; and EF-G, 0.05 to 5 \mu g. The reaction was carried out at 37 C and was terminated by adding aliquots of 0.05 ml to Whatman 3MM filters (25 mm in diameter) to which 0.01 ml of 10% trichloroacetic acid had been added. The filters were immersed in 10% trichloroacetic acid for 10 min and then washed with 5% acid, ethanol-ether (1:1), and ether. After drying, the filters were counted in toluene.

Determination of protein. Determination of protein was by the method of Lowry et al. (13).

Purification of EF-G. When starting from large batches of bacteria (more than 20 g, wet weight, of cells), purification was carried out by the procedures previously used for purification of elongation factors from E. coli (14, 18). To purify EF-G from small batches of cells (less than 2 g, wet weight), we developed a method in which the centrifugation of S-30 extracts at 100,000 \times g and the ammonium sulfate fractionation are replaced by passage through a column of Sepharose 6B. This gel separates an S-30 extract into three fractions: ribosomes and particles larger than 6 \times 10^6 daltons are eluted in the void volume; elongation factors come out in the second fraction, separated from the low-molecular-weight material that comes out in the third fraction. During purification, EF-G activity was determined by three assay methods: (i) ribosomal-dependent GTPase activity (shown in this paper), (ii) ability of EF-G to complement EF-T in the polyuridylic acid-directed synthesis of polyphenylalanine in a B. subtilis cell-free system (Aharonowitz and Ron, submitted for publication), and (iii) net EF-G-derived synthesis of peptideyl-\textsuperscript{3H}puromycin, a reaction discussed in full detail in this paper.

In a typical experiment, 1 g of B. subtilis cells was suspended in 1 ml of TAM buffer that contained 10 \mu g of deoxyribonuclease. The S-30 fraction was prepared as described before and was layered on a column of Sepharose 6B (1.5 by 85 cm) that had been equilibrated with TAM buffer containing 1 mM dithiothreitol. The column was eluted with the same buffer (flow rate, 0.3 ml/min), and two-ml fractions were collected. EF-G activity was eluted in tubes 42 to 48 (Fig. 1). The active fractions were pooled, dialyzed against phosphate buffer, pH 7.0, and applied to a column of hydroxylapatite (Fig. 2). This two-step purification resulted in a 70-fold increase in the specific activity of EF-G.

Chemicals. \textsuperscript{3H}puromycin (specific activity, 3.7 Ci/mmol) and \gamma^{32P}GTP (specific activity, 2.3 Ci/ mmol) were purchased from the Radiochemical Centre (Amersham, England). Sepharose 6B was obtained from Pharmacia Fine Chemicals, and hydroxylapatite (Bio-Gel HTP) was from Bio-Rad Laboratories. Amino acids, nucleotides, polyuridylic acid, dithiothreitol, spermidine, and puromycin dihydrochloride were from Sigma Chemical Co.

RESULTS

Translocation of native peptideyl-tRNA molecules on washed ribosomes. When B. subtilis ribosomes were washed to remove bound EF-G (see next section for details), they still contained peptideyl-tRNA molecules ("native" peptideyl-tRNA) distributed between the A site and the P site of the ribosomes. The peptideyl-tRNA molecules bound at the P site could be released from the ribosomes by the addition of puromycin. This reaction is easily followed by the method of Pestka (21, 22), in which the con-

![Fig. 1. Chromatography of S-30 extract on a column of Sepharose 6B. Two milliliters of S-30 extract (30 mg/ml) was applied to a column of Sepharose 6B as described in the text. Protein (broken line) was determined by the method of Lowry et al. (13). Aliquots (0.01 ml) were assayed for GTPase activity (●).](http://jb.asm.org)
version of $[^3]$H]puromycin to peptidyl$[^3]$H]puromycin is measured by determining trichloroacetic acid-precipitable $[^3]$H counts. However, the fraction of the peptidyl-tRNA molecules that was located at the A site of the ribosome could not react with puromycin until it was moved to the P site by translocation. Thus, the addition of EF-G and GTP to the washed ribosomes stimulated the synthesis of peptidyl-puromycin considerably, suggesting that the majority of the native peptidyl-tRNA molecules was located at the A site on the washed ribosomes (Fig. 3A). Experimental values for the fraction of peptidyl-tRNA molecules in the A site varied from 30 to 60%. It should be noted that the formation of peptidyl-puromycin involving peptides at the P site levels off faster than the process involving translocation, presumably due to the greater complexity of the latter. The results presented in Fig. 3B indicate that the stimulation of peptidyl-puromycin synthesis by EF-G is GTP dependent. These results suggest that the increase in the synthesis of peptidyl-puromycin brought by the addition of EF-G ("EF-G-derived synthesis of peptidyl-puromycin") can serve as a measure for the level of translocation in this system. In the following experiments, this reaction is further characterized.

Washed ribosomes as donors for EF-G-derived synthesis of peptidyl-puromycin. In E. coli, EF-G remains tightly bound to the ribosomes during purification and is removed only after several washings in high salt concentrations (9). A similar situation exists in B. subtilis since the addition of GTP alone to unwashed ribosomes increased the synthesis of peptidyl-puromycin by 60%, and no further increase could be detected after the addition of EF-G (Table 1). Three washings in buffer containing 1 M ammonium chloride were necessary to remove the EF-G from the ribosomes, as indicated by the inability of GTP to stimulate the reaction without the addition of EF-G. After three washings, the combined addition of GTP and EF-G stimulated peptidyl-puromycin formation by 80%, and no further stimulation was achieved by additional washings. It should

![Figure 2](image-url)  
**Fig. 2.** Elution of EF-G activity (from Sepharose 6B) from a column of hydroxylapatite. Protein (23 mg) with EF-G activity (eluted from a column of Sepharose 6B and treated as described in the text) was applied to a column of hydroxylapatite (10-ml bed volume). A stepwise increase in the concentration of phosphate was used to elute the EF-G activity. It was eluted at 40 mM phosphate. Aliquots (0.01 ml) were taken for GTPase activity (●). $A_{280}$ (broken line) is optical density at 280 nm.

![Figure 3](image-url)  
**Fig. 3.** Effect of EF-G on synthesis of peptidyl-$[^3]$H]puromycin by washed ribosomes. For reaction conditions, also see the text. The 0.1-ml reaction mixture contained Tris-hydrochloride, pH 7.8, 50 mM; ammonium chloride, 50 mM; magnesium acetate, 6 mM; spermidine, 2 mM; [H]puromycin, 1.6 x 10$^{-6}$ M (770 counts/min per pmol); washed ribosomes, 3.87 $A_{260}$ units, and GTP, 6.2 x 10$^{-4}$ M. (A) Symbols: △, ribosomes + EF-G (6.3 μg) + EF-T (12.6 μg); ●, ribosomes + EF-G; ○, ribosomes + EF-T; □, ribosomes. (B) Same as in (A), except GTP was omitted.

| Table 1. Effect of NH$_4$Cl washings on the synthesis of peptidyl-puromycin on ribosomes |
|-----------------------------------------------|-----------------|-----------------|
| None | GTP | EF-G | GTP + EF-G |
| 0 | 1.48 | 2.13 | 1.38 | 2.18 | 0.05 |
| 1 | 1.30 | 2.64 | 1.29 | 2.67 | 0.03 |
| 2 | 1.42 | 2.35 | 1.37 | 2.71 | 0.36 |
| 3 | 1.31 | 1.28 | 1.24 | 2.41 | 1.13 |
| 4 | 1.23 | 1.16 | 1.30 | 2.16 | 1.00 |
be noted that the salt washings removed less than 20% of all the reactive peptidyl-tRNA molecules bound to the ribosomes. There is at least one such reactive molecule per 10 ribosomes, as calculated by assuming that 1 A_{260} unit is equivalent to 25 pmol of 70S ribosomes and taking 2.5 pmol of peptidyl-puromycin formed per A_{260} unit as the average activity.

Effect of ribosome concentration on EF-G-derived synthesis of peptidyl-puromycin. The addition of EF-G and GTP to washed ribosomes stimulated both the rate and the extent of the formation of peptidyl-puromycin over a wide range of ribosome concentrations. The results in Fig. 4 indicate that the rate of EF-G-derived synthesis of peptidyl-puromycin increased linearly with ribosome concentration up to 4 A_{260} units, whereas the extent of the stimulation was linear with ribosome concentrations up to 10 A_{260} units (Fig. 5).

Interaction of EF-G and GTP with ribosomes in the presence and absence of puromycin. The results presented above suggest that the stimulation of peptidyl-puromycin synthesis by EF-G and GTP can be used as a measure of translocation. However, since puromycin was always present in the reaction mixture, it was necessary to show that the translocation event takes place independently of the presence of puromycin. This was shown in the following experiment. Samples of washed ribosomes were incubated with GTP, with EF-G, or with GTP and EF-G for 5 min. The samples were then passed through a small column (0.5 by 2 cm) of Sepharose 6B to remove the low-molecular-weight components. Puromycin was then added to each of the ribosome fractions, and the formation of peptidyl-puromycin was assayed. The results (Fig. 6) indicate that preincubation with EF-G and GTP was the only kind of treatment

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**Fig. 4.** Effect of ribosome concentration on the rate of synthesis of peptidyl-puromycin. Reaction was for 1 min in 0.07 ml, containing EF-G at a final concentration of 1.15 \( \mu \text{g/tube} \). Other components were Tris-hydrochloride, pH 7.8, 10 mM; magnesium acetate, 4 mM; ammonium chloride, 100 mM; GTP, \( 6 \times 10^{-4} \text{M} \); and \(^{3}H\)-puromycin, 96 pmol (760 counts/min per pmol). (A) Reaction rate with (\( \bigcirc \)) and without (\( \bigcirc \)) EF-G. (B) EF-G-derived synthesis of peptidyl-puromycin. This curve was obtained from the difference of the two curves in (A).

**Fig. 5.** Effect of ribosome concentration on the extent of synthesis of peptidyl-\(^{3}H\)-puromycin. Same as in Fig. 4, except incubation was for 10 min.

**Fig. 6.** Effect of preincubation of ribosomes with EF-G and GTP on the subsequent synthesis of peptidyl-puromycin. The incubation reaction contained, in 0.1 ml, Tris-hydrochloride, pH 7.8, 10 mM; magnesium acetate, 4 mM; ammonium chloride, 100 mM; potassium chloride, 8 mM; and ribosomes (\( \bigcirc \)), ribosomes with GTP (4.3 \( \times 10^{-4} \text{M} \)) (\( \bigcirc \)), ribosomes with EF-G (1.5 \( \mu \text{g} \)) (\( \bigtriangleup \)), or ribosomes with GTP and with EF-G (\( \bigcirc \)). Incubation was for 5 min at 37 \( \text{C} \) and was followed by passage through a small column of Sepharose 6B. The fraction that contained the ribosomes was incubated with \(^{3}H\)-puromycin (90 pmol, 680 counts/min per pmol), and the synthesis of peptidyl-puromycin followed. The values of peptidyl-puromycin synthesized are calculated per 1 A_{260} unit since the recovery of ribosomes varied from tube to tube.
that stimulated the subsequent reaction with puromycin. Moreover, the formation of peptidyl-puromycin by the preincubated ribosomes followed the kinetics previously observed for peptides bound at the A site (see Fig. 3A for comparison). These findings support the assumption that translocation of the peptidyl-tRNA molecules by EF-G took place before, and independently of, the addition of puromycin.

The process of translocation requires the simultaneous presence of both GTP and EF-G on the ribosome, as indicated by the results shown in Fig. 7. In this experiment ribosomes were preincubated with EF-G, with GTP, with both, or with neither. They were then passed through a Sepharose 6B column and incubated with puromycin and the component(s) that was omitted during the preincubation. Translocation occurred only in those samples in which GTP and EF-G could interact with the ribosome at the same time.

**DISCUSSION**

In the present work the activity of EF-G was studied by following the translocation of "native" peptides on ribosomes. The method was based on the finding that one out of ten washed ribosomes of *B. subtilis* carries peptidyl-tRNA molecules bound to one of the two ribosomal binding sites. Only a fraction of these peptidyl-tRNA molecules (about half) can react to give peptidyl-puromycin (Table 1). The other fraction can react with puromycin only after being translocated on the ribosomes by EF-G and GTP. The difference between these two activities represents the actual EF-G-derived translocation in the system. Using [3H]puromycin, which precipitates in trichloroacetic acid only when bound to peptides (21, 22), enables us to follow the reaction with a rather high degree of sensitivity. The methods used in this work to extract EF-G from large quantities of *B. subtilis* were based on those used in *E. coli*. When small quantities of cells were extracted, separation on Sepharose 6B was adopted (see above). Although it has been shown that preparations of *E. coli* EF-G are contaminated with enzymes utilizing GTP and guanosine 5'-diphosphate as substrates (23), we nevertheless found the GTPase reaction to be useful in following the progress of our purification steps of *B. subtilis* EF-G, due to its simplicity and once we had verified that concomitant activities, such as polymerization of phenylalanine and peptidyl-[3H]puromycin synthesis, occurred in the same fractions. The system described here has made it possible to make the first observations on the interaction of *B. subtilis* EF-G, ribosomes, and GTP in translocation. The results represented in Fig. 6 and 7 show that neither GTP nor EF-G can form a stable complex with the ribosome. Peptidyl-[3H]puromycin can be formed only when both GTP and EF-G are present simultaneously. These results are in agreement with those of Bodley et al. (2) that EF-G and GTP form a stable complex only when present together with the antibiotic fusidic acid. When fusidic acid was added in a final concentration of 3 mM to our system, the reaction could not exceed the extent of the controls without the addition of EF-G (results not shown).

The synthesis of peptidyl-[3H]puromycin, mediated by EF-G in a *B. subtilis* system, was found to be similar to that of *E. coli* polyribosomes in its optimal response to NH₄⁺ and Mg²⁺ concentrations, 100 to 150 mM and 4 mM, respectively (21). In addition, EF-G of *E. coli* was capable of replacing *B. subtilis* EF-G in the in vitro synthesis of polyphenylalanine by extracts of the latter (1).

This study of EF-G-dependent synthesis of peptidyl-[3H]puromycin by the use of native peptide donors offers a tool for following translocation in an in vitro system of *B. subtilis*, closer to the conditions of the intact organism than most of the other systems thus far developed. An additional advantage of using native peptide donors for studying translocation is its convenience. The only preparation step required is the washing of the ribosomes.

![Fig. 7. Interaction between ribosomes, EF-G, and GTP. The experiment was carried out as in Fig. 6, except the [3H]puromycin incubation mixture contained the components that had been omitted during the preincubation (shown in the figure).](http://jb.asm.org/)
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