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Elongation Factor Ts from the Antarctic Eubacterium *Pseudoalteromonas haloplanktis* TAC 125: Biochemical Characterization and Cloning of the Encoding Gene[†,‡]

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ABSTRACT: The elongation factor Ts was isolated from the psychrophilic Antarctic eubacterium *Pseudoalteromonas haloplanktis* TAC 125 strain (*Ph*EF-Ts), and its functional properties were studied. At 0 °C *Ph*EF-Ts enhanced the [^{3}H]GDP/GDP exchange rate on the preformed *Ph*EF-Tu·[^{3}H]GDP complex by 2 orders of magnitude even at very low Tu:Ts ratio, by lowering the energy of activation of the exchange reaction. *Ph*EF-Ts is a monomeric protein, and in solution it forms a stable dimeric complex with *Ph*EF-Tu. The *Ph*EF-Ts encoding gene was cloned and sequenced. Its structural organization was similar to that of *Escherichia coli* because it showed at its 5′ end the gene encoding the ribosomal protein S2. The translated amino acid sequence had a calculated molecular weight of 30762, and showed a high sequence identity with *E. coli* (68%) and *Thermus thermophilus* (44%) EF-Ts. The *Ph*EF-Ts primary structure contains well-preserved almost all the amino acid residues interacting at the interfaces of the *E. coli* EF-Ts·EF-Tu complex. Finally, the high concentration of *Ph*EF-Ts in this psychrophilic eubacterium might represent an adaptive tool to ensure an efficient nucleotide exchange even at low temperature.

In the course of protein synthesis in eubacteria, the elongation factor Tu (EF-Tu)[1] in its active GTP-bound form delivers the aminoacyl-tRNA to the mRNA-programmed A-site of bacterial ribosome. EF-Tu is a representative member of the GTPase superfamily (*1*), and its function is driven by the GTP hydrolysis that leads to the inactive GDP-bound form of EF-Tu, which then dissociates from the ribosome (*2*). The regeneration of active EF-Tu is mediated by the guanine nucleotide exchange factor Ts (EF-Ts), which displaces GDP from the EF-Tu·GDP complex, thus allowing the formation of a new ternary complex, EF-Tu·GTP·aa-tRNA (*3*). Therefore, EF-Ts greatly accelerates the nucleotide exchange on EF-Tu, and its exchange function is associated with the relative amount of EF-Ts and EF-Tu present in *Escherichia coli* cells, since the affinity of EF-Tu for GDP is 2 orders of magnitude higher than that for GTP (*3*). EF-Ts, first discovered in *Pseudomonas fluorescens* (*4*), has been isolated also from several other sources. We have previously described the functional and structural properties of its archaeal counterpart (EF-1β) isolated from the hyperthermophile *Sulfolobus solfataricus* (*5*). Insight into the nucleotide exchange mechanism has been derived from the determination of the 3D structure of the EF-Tu·EF-Ts complex in *E. coli* (*6*) and *Thermus thermophilus* (*7*, *8*). However, some differences characterize the mechanism of the EF-Tu·nucleotide exchange promoted by EF-Ts in these two systems (*7*, *8*).

Recently, attention has been devoted to enzymes isolated from psychrophilic organisms (*9*, *10*) because of their ability to catalyze reactions with high efficiency at very low temperature (*11*). They show a greater flexibility of the protein structure compared to that of the mesophilic or thermophilic counterparts, allowing conformational changes that favor the enhancement of the catalytic rate. However, as a consequence of the higher flexibility, these enzymes also undergo a faster thermal inactivation (*9*−*12*). In this work, we describe the molecular and biochemical characterization of EF-Ts isolated from the psychrotolerant Antarctic eubacterium *Pseudoalteromonas haloplanktis* TAC 125 (*Ph*), formerly called *Moraxella* TAC 125 (*13*), whose growth temperature is in the range 4−20 °C. In addition, the molecular interaction between *Ph*EF-Ts and *Ph*EF-Tu, in different experimental conditions, has been analyzed.

EXPERIMENTAL PROCEDURES

Materials. DEAE-Sepharose (fast flow), Superdex 75 HR 10/30, HiLoad Superdex 75 (2.6 × 60 cm), Mono Q HR

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[‡] The nucleotide sequence of the *Ph*EF-Ts gene has been deposited at the EMBL database under the accession number AJ628730.

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[1] Abbreviations: *Ph*, *Pseudoalteromonas haloplanktis*; *Ec*, *Escherichia coli*; *Tt*, *Thermus thermophilus*; *Ss*, *Sulfolobus solfataricus*; EF-, elongation factor.

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10/10, and [^{3}H]GDP were from Amersham; GDP and GTP were from Boehringer Mannheim. Electrophoretic materials were from Bio-Rad.

The following buffers were used: (buffer A) 20 mM Tris·HCl, pH 7.8, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 10% (v/v) glycerol; (buffer B) 20 mM Tris·HCl, pH 7.8, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 50 mM KCl, 10% (v/v) glycerol.

Restriction enzymes, modifying enzymes, labeled compounds, and chemicals were as already reported (*14*); plasmid DNA, genomic DNA, and labeled probes were prepared according to standard procedures (*15*). *Ph*EF-Tu was isolated as reported (*14*).

Assay of PhEF-Ts Activity. During the purification steps, the presence of *Ph*EF-Ts was checked by its ability to accelerate the formation of the *Ph*EF-Tu·[^{3}H]GDP complex. Unless otherwise indicated the standard reaction mixture contained 0.3−1.0 μM *Ph*EF-Tu, 2.5 μM [^{3}H]GDP (s.a. 300−600 cpm/pmol), and an appropriate amount of *Ph*EF-Ts in 50 μL of buffer B; after incubation for 1 min at 0 °C, 40 μL of aliquot was transferred onto a nitrocellulose filter which was then washed twice with buffer B. The filters were dried and counted for radioactivity. The specific activity of *Ph*EF-Ts was checked by its ability to accelerate the rate of exchange of [^{3}H]GDP for GDP on the preformed *Ph*EF-Tu·[^{3}H]GDP complex. The latter was obtained at 15 °C as reported (*14*) by incubating 21.2 μM *Ph*EF-Tu with 96 μM [^{3}H]GDP (s.a. 875 cpm/pmol) in 125 μL of buffer B. After 1 h of incubation required to reach the equilibrium, the sample was cooled in ice and loaded onto a Superdex 75 HR 10/30 equilibrated at 0.5 mL/min in buffer B, to remove the unbound [^{3}H]GDP excess. Fractions of 250 μL were collected, and those containing radioactive *Ph*EF-Tu were pooled together and checked for purity by SDS−PAGE; the concentration of the *Ph*EF-Tu·[^{3}H]GDP complex was determined by counting the radioactivity.

The rate of exchange of [^{3}H]GDP for GTP on the *Ph*EF-Tu·[^{3}H]GDP complex observed in the absence or in the presence of *Ph*EF-Ts (k_{obs}) was calculated according to a first-order kinetics equation, $\ln(C_t/C_o) = -k_{obs}t$, where C_o is the initial concentration of *Ph*EF-Tu·[^{3}H]GDP and C_t is its concentration at time t. For other experimental details see the caption for Figure 2.

Purification of PhEF-Ts. *Ph*EF-Ts was isolated from a post-ribosomal supernatant (S-100) of a *P. haloplanktis* TAC 125 cell paste, which was prepared as reported (*14*). The S-100 fraction was dialyzed against buffer A and applied to a DEAE-Sepharose fast-flow column (2.5 × 70 cm) equilibrated at 100 mL/h with buffer A. After washing with the same buffer, a linear 0−300 mM KCl gradient in buffer A (4 L total) was applied. The fractions revealing *Ph*EF-Ts activity eluted at about 50 mM KCl were pooled together, dialyzed against buffer A, and then loaded onto a Mono Q HR 10/10 column equilibrated at 3 mL/min with buffer A. The bound proteins were eluted with a linear 0−100 mM KCl gradient in buffer A (200 mL total volume). Fractions containing *Ph*EF-Ts activity were concentrated with Aquacide II and loaded onto a HiLoad Superdex75 gel filtration column (2.6 × 60 cm) equilibrated at 60 mL/h with buffer B. The fractions containing *Ph*EF-Ts activity were analyzed by SDS−PAGE, and only those showing a single protein band were pooled together, concentrated, dialyzed against buffer A containing 50% (v/v) glycerol, and stored at −20 °C. Under these conditions *Ph*EF-Ts was stable for at least one year. Following this procedure, about 33 mg of pure *Ph*EF-Ts can be obtained from 110 g of cell paste homogenate, accounting for at least 6% of the total protein.

Determination of the Molecular Weight of PhEF-Ts and the PhEF-Tu·PhEF-Ts Complex. The molecular weight of *Ph*EF-Ts and of the *Ph*EF-Tu·*Ph*EF-Ts complex under native conditions was estimated by gel filtration performed on Superdex 75 HR 10/30 connected to a computer-assisted FPLC apparatus (Pharmacia). The column was equilibrated at room temperature with buffer B at a flow rate of 0.5 mL/min and calibrated using 10 μg in 25 μL of the following protein standards, run both separately and in combination: human transferrin (M_r 80000), bovine serum albumin (M_r 69000), ovalbumin (M_r 46000), and carbonic anhydrase (M_r 30000). The void volume was determined using Dextran Blue 2000.

The molecular weight of *Ph*EF-Ts under denaturing conditions was determined by SDS−PAGE performed in the presence of β-mercaptoethanol, using a 12% polyacrylamide gel; after the electrophoretic run, the gels were stained by Coomassie Brilliant Blue G250. Standard proteins were as reported above, including soybean trypsin inhibitor (M_r 21000).

Determination of the N-Terminal Amino Acid Sequence of PhEF-Ts. The N-terminal amino acid sequence of the purified *Ph*EF-Ts was determined by stepwise Edman degradation in an automated protein sequencer (Applied Biosystem), using an electroblotted protein sample on a PVDF membrane (Amersham).

Evaluation of the Thermal Stability of PhEF-Tu. The heat inactivation of *Ph*EF-Ts was investigated by incubating 0.6 mg/mL protein in buffer B for 10 min at different temperatures in the interval 15−90 °C. After the heat treatment the mixtures were cooled on ice for at least 30 min and then assayed for the residual GDP/[^{3}H]GDP exchange activity on *Ph*EF-Tu. In addition, the heat denaturation of *Ph*EF-Ts was evaluated by UV melting curves by measuring the difference $A_{274} - A_{286}$ in the temperature range 15−80 °C (*16*, *17*).

Formation of the PhEF-Tu·PhEF-Ts Complex. *Ph*EF-Tu·[^{3}H]GDP (520 pmol), prepared as described above, was incubated in 110 μL of buffer B with about 1 nmol of purified *Ph*EF-Ts for 1 h at 0 °C. The reaction mixture was then analyzed on a Superdex75 HR 10/30 gel filtration column, equilibrated, and calibrated as described above.

Cloning the PhEF-Ts Encoding Gene. *P. haloplanktis* TAC 125 genomic DNA was used as a template to synthesize by PCR a DNA fragment containing part of the EF-Ts gene. The forward primer ACiGGiGCiGGiATGATGGA(T,C)TG-(T,C,)AA (TS1) was designed from the N-terminal amino acid sequence determined on the purified protein and corresponding to the peptide T15GAGMMDCK, whereas TS3 (reverse primer, GCiGCiAC(G,A)TGCATiGCiAT-(G,A)TG(T,C)TT) corresponded to the peptide sequence K166HIAMHVAA (as numbered in *E. coli* EF-Ts) (*18*) highly conserved among many eubacterial EF-Ts's. These primers contained inosine (i) to cover all synonymous codon positions. The PCR product (0.5 kbp) was cloned in pGEM T-easy plasmid and sequenced. The recombinant plasmid was then used as a probe to analyze two mini *P. haloplanktis* DNA libraries obtained by cloning into the pGEM T-easy

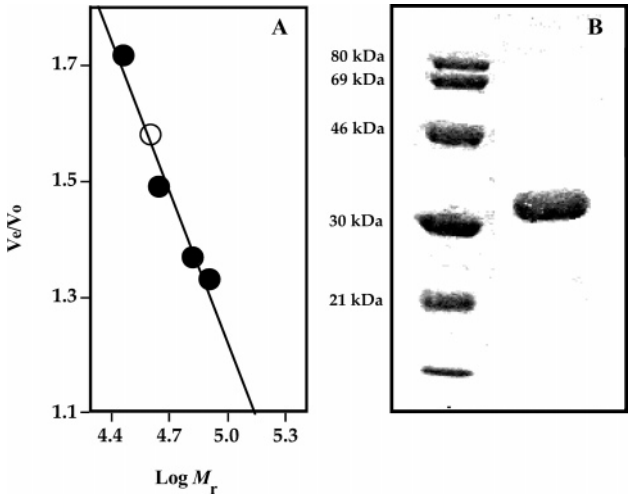


FIGURE 1: Molecular organization of *Ph*EF-Ts. (A) Elution on gel filtration. The column was equilibrated and calibrated as described in the text. The elution is indicated with filled circles for M_r standards and with an empty circle for *Ph*EF-Ts. (B) SDS−PAGE of *Ph*EF-Ts. Lane A: M_r markers (the size is indicated on the left). Lane B: electrophoretic behavior of 4 μg of pure *Ph*EF-Ts.

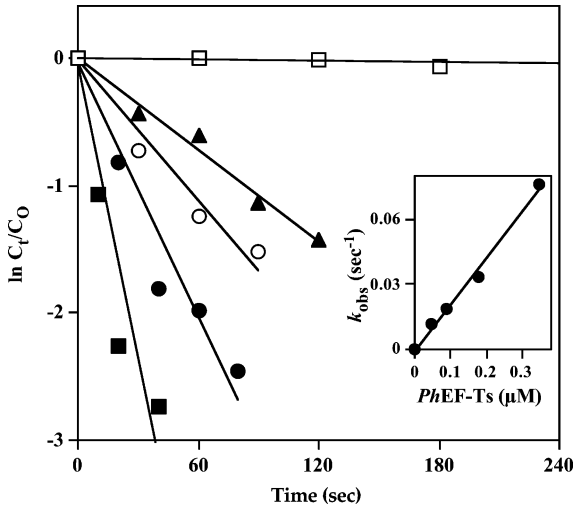


FIGURE 2: Effect of increasing amounts of *Ph*EF-Ts on the [^{3}H]-GDP/GDP exchange on the *Ph*EF-Tu·[^{3}H]GDP complex. In a final volume of 170 μL of buffer B, 1.5 μM *Ph*EF-Tu·[^{3}H]GDP (s.a. 6400 cpm/pmol) was incubated at 0 °C with 400 μM GDP in the absence (□) or in the presence of 0.048 (▲), 0.09 (○), 0.18 (●), and 0.35 μM (■) *Ph*EF-Ts, respectively. At the times indicated, 30 μL aliquots were filtered onto nitrocellulose and the radioactivity was determined. The data were analyzed according to a first-order kinetics. Inset: The dissociation rate constants (k_{obs}) were plotted against *Ph*EF-Ts concentration.

vector PCR fragments amplified from genomic *Ph*DNA, using in one reaction as the forward primer TS4 (AATAT-CGCTGCTGAAGGCGCTAT, corresponding to peptide N54IAAEGAI) and as the reverse primer pdN6 and in the other reaction pdN6 as the forward primer and TS5 as the reverse primer (TCACCCGTGATGTACTGTAGACG, corresponding to peptide R132LQYITGE). Two positive recombinant clones were selected and sequenced.

Other Methods. SDS−PAGE was carried out on 12% polyacrylamide gels (*19*). The three-dimensional modeling of *Ph*EF-Ts was carried out with the help of the SwissModel server (*20*) using the crystal structure coordinates of EF-Ts from *E. coli* (*6*) as a template (PDB entry 1EFU). Proteins were estimated by the method of Lowry (*21*) using bovine serum albumin as a standard. Sequence analysis and alignment were established with the help of a protein analysis software system packed with the programs PALIGN (*22*) and CLUSTAL (*23*).

Table 1: Effect of Temperature on the GDP/[^{3}H]GDP Exchange on the *Ph*EF-Tu·GDP Complex[a]

T (°C)	exchange rate without *Ph*EF-Ts (pmol of [^{3}H]GDP bound·s^{-1})	exchange rate in the presence of *Ph*EF-Ts (pmol of [^{3}H]GDP bound·s^{-1})
0	0.08×10^{-2}	2.11×10^{-2}
5	0.11×10^{-2}	2.50×10^{-2}
10	0.15×10^{-2}	2.85×10^{-2}
15	0.27×10^{-2}	3.46×10^{-2}
20	0.35×10^{-2}	3.78×10^{-2}
25	0.62×10^{-2}	nd
30	1.12×10^{-2}	nd
35	1.85×10^{-2}	nd

[a] A 400 μL sample of buffer B containing 2.3 μM *Ph*EF-Tu·GDP was incubated with 10 μM [^{3}H]GDP (s.a. 450 cpm/pmol) at the indicated temperatures either in the absence or in the presence of 0.04 μM *Ph*EF-Ts. At appropriate time intervals, 90 μL aliquots were withdrawn and the formed *Ph*EF-Tu·[^{3}H]GDP was determined.

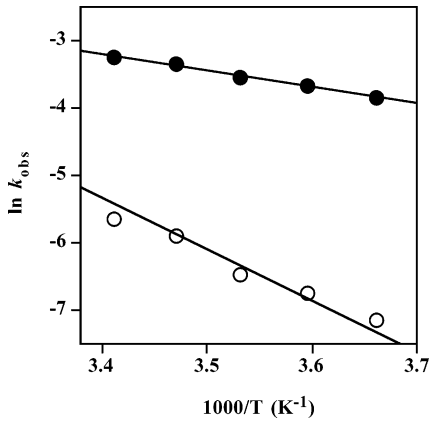


FIGURE 3: Temperature effect on the [^{3}H]GDP/GDP exchange on the *Ph*EF-Tu·GDP complex. The k_{obs} for the [^{3}H]GDP/GDP exchange reaction, in the absence (○) or in the presence (●) of *Ph*EF-Ts was derived from the data reported in Table 1 and analyzed according to the Arrhenius equation.

RESULTS

Molecular and Functional Properties of PhEF-Ts. The molecular weight of purified *Ph*EF-Ts was determined under either native or denaturing conditions. Analyzed by gel filtration, native *Ph*EF-Ts revealed a molecular weight of about 38000 (Figure 1A), whereas, as shown in Figure 1B, analyzed by SDS−PAGE, *Ph*EF-Ts showed a molecular weight of 33000. These behaviors indicated that *Ph*EF-Ts is a monomeric protein; the slightly higher molecular weight found on native gel filtration could depend on the less compact conformation of the cold-adapted EF-Ts with respect to the mesophilic protein used for the calibration of the column.

The activity of *Ph*EF-Ts was analyzed by the evaluation of its ability to accelerate the rate of the [^{3}H]GDP/GDP exchange reaction on the preformed *Ph*EF-Tu·[^{3}H]GDP complex. As shown in Figure 2, at 0 °C the exchange reaction in the absence of *Ph*EF-Ts was quite slow. The addition of the exchange factor considerably increased the rate of the [^{3}H]GDP/GDP exchange, and the inset of Figure 2 shows

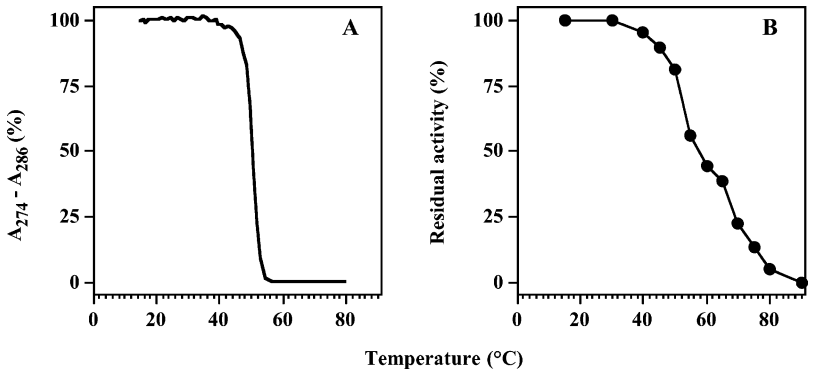


FIGURE 4: Heat stability of *Ph*EF-Ts. (A) UV melting curve of a 19.8 μM *Ph*EF-Ts sample. (B) Heat inactivation profile. A 19.8 μM concentration of *Ph*EF-Ts was incubated in a 70 μL final volume of buffer B in a stoppered tube at temperatures ranging from 15 to 90 °C. After 10 min of incubation, the tubes were cooled on ice and diluted with 630 μL of buffer B and the residual nucleotide exchange activity was followed kinetically at 0 °C as described in the Experimental Procedures. The results are expressed as the percent of the activity of a *Ph*EF-Ts sample kept at 0 °C throughout the experiment.

that, at an EF-Tu:EF-Ts ratio ranging from 30:1 to 5:1, a linear relationship between the amount of *Ph*EF-Ts added and the rate of the [^{3}H]GDP/GDP exchange reaction on the *Ph*EF-Tu[^{3}H]GDP complex was found.

Effect of Temperature on the PhEF-Ts Nucleotide Exchange Activity. The activity of *Ph*EF-Ts was evaluated at different temperatures, measuring the rate of [^{3}H]GDP binding by *Ph*EF-Tu at selected temperatures, either in the absence or in the presence of *Ph*EF-Ts. The results reported in Table 1 showed that the stimulation of the [^{3}H]GDP binding of *Ph*EF-Tu increased with increasing temperatures up to 20 °C. Above this temperature no effect on the *Ph*EF-Ts activity was detectable because all the *Ph*EF-Tu used in the experiment was completely titrated by [^{3}H]GDP within 1 min, even in the absence of *Ph*EF-Ts.

The rate of the [^{3}H]GDP/GTP exchange on the *Ph*EF-Tu·[^{3}H]GDP complex (k_{obs}) was evaluated in the temperature range 0−20 °C either in the absence or in the presence of *Ph*EF-Ts; in both cases, the dissociation rate constant increased with increasing temperature. The corresponding Arrhenius plots were linear in the 0−20 °C interval. The energy of activation (*E*) of the [^{3}H]GDP/GDP exchange reaction calculated from the Arrhenius equation was 63 and 20 kJ/mol in the absence and in the presence of the exchange factor, respectively. These results indicated that the enzymatic role of *Ph*EF-Ts consists of lowering the energy of activation of the exchange reaction, thus making faster the nucleotide exchange process.

Heat Stability of PhEF-Ts. To investigate the heat resistance of the psychrophilic nucleotide exchange factor, we have followed the heat denaturation of *Ph*EF-Ts by UV melting curves in the interval 15−80 °C. The results reported in Figure 4A indicate that the temperature for half-denaturation of the protein was around 50 °C. Another approach used to study the heat resistance of *Ph*EF-Ts consisted in the evaluation of the residual *Ph*EF-Ts activity after its exposure for 10 min at different temperatures, ranging from 0 to 90 °C (Figure 4B). In this case the temperature for half-inactivation was about 57 °C. It is remarkable that in both cases the temperature measured to half-inactivate/denaturate *Ph*EF-Ts was quite high with respect to the optimal growth temperature of this psychrotolerant microorganism.

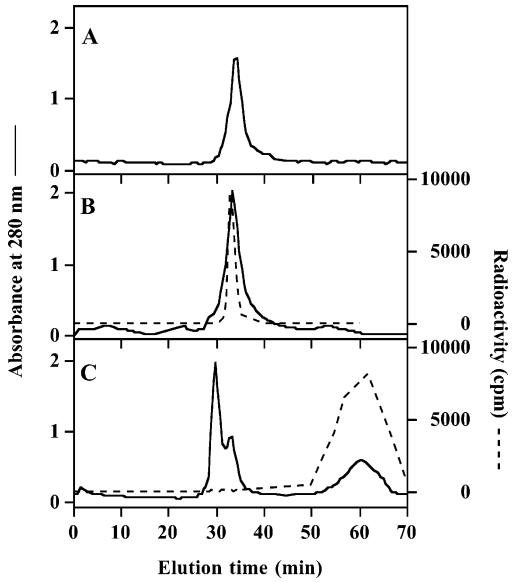
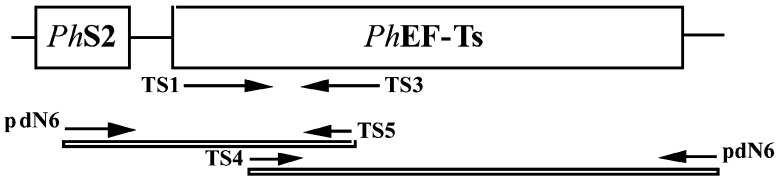


FIGURE 5: Formation of the *Ph*EF-Tu·*Ph*EF-Ts complex. The samples were prepared as described in the Experimental Procedures and then loaded onto a Superdex75 HR 10/30 gel filtration column, equilibrated as described in the text. Proteins eluted were monitored at 280 nm (continuous line). Fractions of 250 μL were collected, and the radioactivity was counted on 100 μL aliquots (dashed lines). (A) Elution profile of a pure *Ph*EF-Ts sample. (B) Elution profile of a *Ph*EF-Tu·[^{3}H]GDP sample. (C) Elution profile of a reaction mixture containing *Ph*EF-Tu·[^{3}H]GDP and *Ph*EF-Ts after 1 h of incubation at 0 °C.

PhEF-Tu·PhEF-Ts Complex. The interaction between the nucleotide exchange factor and *Ph*EF-Tu was studied by gel filtration. Figure 5A shows the elution profile of purified *Ph*EF-Ts (M_r 38000), whereas Figure 5B shows that of the preformed radiolabeled *Ph*EF-Tu·[^{3}H]GDP complex (M_r 47500) (*14*). The incubation for 1 h at 0 °C of *Ph*EF-Tu·[^{3}H]GDP with a 2 M *Ph*EF-Ts excess led to the formation of a new protein peak, eluting at a position accounting for an M_r of about 80 000 (Figure 5C). This finding suggests that the *Ph*EF-Tu·*Ph*EF-Ts complex should display a 1:1 stoichiometry. In addition, all the radioactivity was collected as free [^{3}H]GDP; because under these conditions *Ph*EF-Tu·[^{3}H]GDP incubated in the absence of *Ph*EF-Ts did not dissociate (Figure 5B), the shift of the radioactive peak observed in Figure 5C was due to the addition of *Ph*EF-Ts.

A



B

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 E  T  L  K  H  V  A  M  H  V  A  A  S  S  P  E  Y  L  T  P 180
TCAGACGTTCCTGCTGATGTTGTAGCTAAAGAACAACAAGTACAAATCGAAATCGCGATG  600
 S  D  V  P  A  D  V  V  A  K  E  Q  Q  V  Q  I  E  I  A  M 200
AATGAAGGTAAATCTGCTGAAATCGCTGAGAAGATGGTTGTTGGCCGCATGAAAAAATTC  660
 N  E  G  K  S  A  E  I  A  E  K  M  V  V  G  R  M  K  K  F 220
ACTGGTGAGGTTTCTCTTACTGGTCAAGCTTTCATCATGGAACCTAAGAAAACTGTTGGC  720
 T  G  E  V  S  L  T  G  Q  A  F  I  M  E  P  K  K  T  V  G 240
GAAATTCTTAAAGAGAAAAACGCAACAGTTACTAGCTTCGTACGTGTAGAAGTTGGTGAA  780
 E  I  L  K  E  K  N  A  T  V  T  S  F  V  R  V  E  V  G  E 260
GGTATCGAAAGAAAAGAAGAAGATTTTGCTGCTGAAGTTGCTGCACAAATCGCTGCGTCA  840
 G  I  E  R  K  E  E  D  F  A  A  E  V  A  A  Q  I  A  A  S 280
AGCTTTCATCATGGAACCTAAgaaaactgttggcgaaattcttaaagagaaaaacgcaac  900
 S  F  H  H  G  T  •                                         286
Agttactagcttcgtacgtgtagaagttggtgaaggtatcgaaagaaaagaagaagattt  960
```

FIGURE 6: Sequence of the gene encoding *Ph*EF-Ts. (A) Sequencing strategy. Arrows indicate the primers utilized for PCR. (B) Nucleotide sequence of the *Ph*EF-Ts gene with its flanking regions and deduced amino acid sequence. The putative ribosomal binding site is in italics. The potential promoter element, palindromic sequence, and transcription termination signal are underlined. The sequences of the regions corresponding to the PCR primers are underlined and reported in the order TS1, TS4, TS5, and TS3. The amino acid sequence determined at the N-terminal of the protein is also underlined. The nucleotide sequence and the deduced amino acid sequence of the C-terminal part of *Ph*S2 are also reported (nucleotides −421 to −107).

Therefore, this finding also shows that *Ph*EF-Tu cannot bind GDP and its nucleotide exchange factor simultaneously.

Sequencing of the PhEF-Ts Encoding Genes. The nucleotide sequences of the two overlapping clones (see the Experimental Procedures) gave the complete sequence of the *Ph*EF-Ts encoding gene. Figure 6 reports the nucleotide sequence of the gene encoding *Ph*EF-Ts and its flanking regions. The 5′ untranslated region is 31 bp long and contains a potential ribosomal binding site at position −12. This region separates the *Ph*EF-Ts from the gene encoding the ribosomal protein S2 (*rps2*). To determine the size of the transcript containing the *Ph*EF-Ts gene, total RNA from *P. haloplanktis* was analyzed by Northern blot. Only one transcript was detected corresponding to an RNA of about

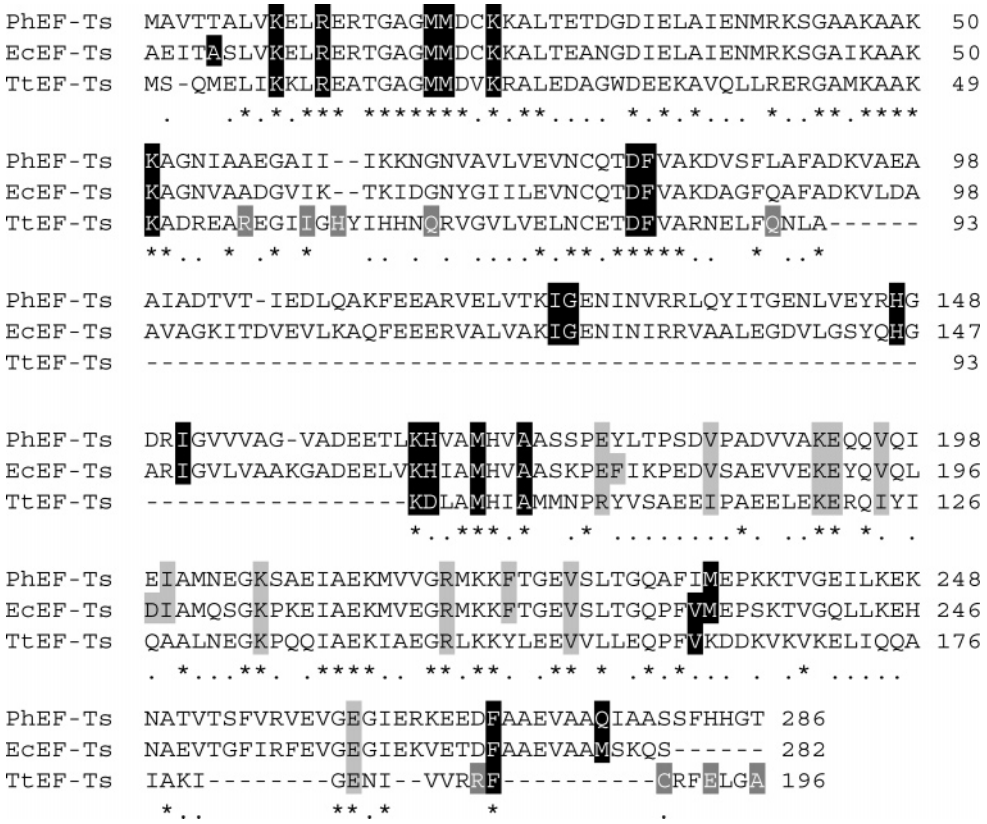

FIGURE 7: Alignment of the amino acid sequences of *Ph*EF-Ts, *Ec*EF-Ts, and *Tt*EF-Ts. Asterisks and dots indicate sequence identities and similarities, respectively. Identical residues that in *Ec*EF-Ts are involved in the dimerization domain are gray shadowed, whereas those involved in the interaction with *Ec*EF-Tu are black shadowed. Residues that in *Tt*EF-Ts are involved in its dimerization are gray shadowed and indicated in white.

2.5 kb (not shown), thus suggesting that in *P. haloplanktis* the *Ph*EF-Ts gene is part of a single transcriptional unit also containing the *rps2* gene.

The *Ph*EF-Ts gene encoded a protein made of 286 amino acid residues including the initial methionine, corresponding to a calculated M_r of 30 762. The alignment of *Ph*EF-Ts with *Ec*EF-Ts and *Tt*EF-Ts (*24*) reported in Figure 7 showed sequence identity of 68.5 and 44%, respectively. A 3D model of *Ph*EF-Ts, obtained by using the X-ray coordinates of *Ec*EF-Ts complexed with *Ec*EF-Tu as a template, was almost superimposable on the template (not shown).

DISCUSSION

This paper is the first report of a translation elongation factor Ts isolated from a psychrophilic eubacterium. *Ph*EF-Ts was purified and identified as the guanosine nucleotide exchange factor of *Ph*EF-Tu as it was able to accelerate the [^{3}H]GDP/GDP exchange on the *Ph*EF-Tu·[^{3}H]GDP complex; in addition, its primary structure showed high sequence homology with that of other eubacterial EF-Ts's.

*Ph*EF-Ts was purified as a monomeric protein as already found for the same enzyme isolated from *E. coli* (*25*), bovine mitochondria (*26*), and *Euglena gracilis* (*27*). Under non-denaturing conditions *Ph*EF-Ts exhibited an M_r of 38000, a value slightly higher than that calculated from the amino acid sequence (30672). This difference can be ascribed to the different flexibility of psychrophilic proteins with respect to the mesophilic ones (*12*, *28*). In fact, such a flexibility makes the volume of the protein larger than that of the less flexible mesophilic or thermophilic ones (*29*). This observation is supported by the finding that the average hydrophobicity for the amino acid residue (*30*) for *Ph*EF-Ts was 4.2 kJ/amino acid, a value lower than that of 4.3 and 4.6 kJ/amino acid calculated for mesophilic *E. coli* EF-Ts and thermophilic *T. thermophilus* EF-Ts, respectively. The higher flexibility of *Ph*EF-Ts can also explain the reduced heat stability of the factor, compared to that of *Ec*EF-Ts (*25*) and *Tt*EF-Ts (*31*, *32*). However, the temperature for half-inactivation/denaturation of *Ph*EF-Ts (57/50 °C) was at least 30 °C higher than the growth temperature of *P. haloplanktis*, a property already found for *Ph*EF-Tu (*14*).

Concerning the catalytic mechanism of *Ph*EF-Ts, the data reported in this paper indicated that the action of *Ph*EF-Ts involved a lowering of the energy of activation of the nucleotide exchange reaction on *Ph*EF-Tu. In addition, the incubation of *Ph*EF-Tu·[^{3}H]GDP with *Ph*EF-Ts led to the formation of a *Ph*EF-Tu·*Ph*EF-Ts complex and the simultaneous release of [^{3}H]GDP, thus indicating that *Ph*EF-Tu cannot bind both ligands at the same time.

In the genome of *P. haloplanctis*, the EF-Ts gene is present in only one copy and its structural organization is similar to that present in *E. coli* (*33*). In fact, also in *P. haloplanktis*, the gene coding for *Ph*EF-Ts (*tsf*) is preceded by the gene encoding the ribosomal protein S2 (*rps2*). These two genes are transcribed as a single unit as suggested by the size of the transcript, thus indicating the presence of a promoter region at the 5′ end of *rps2*. The synonymous codons most frequently used in *Ph*EF-Ts were those ending in A and T.

For instance, among the 33 Val residues, 21 are encoded by GTT, 10 by GTA, and only 2 by GTG. A similar behavior was also observed in the case of the *Ph*EF-Tu gene (*14*). The translated amino acid sequence of *Ph*EF-Ts showed sequence identity with a large number of eubacterial EF-Ts's. A dendrogram derived from the multiple sequence alignment of several EF-Ts sequences showed that psychrophilic *P. haloplanktis* protein shows the highest sequence homology (77% amino acid identity and 9% conservative substitutions) with EF-Ts from *Shewanella oneidensis* (accession no. Q8EGH4), a microorganism belonging to the *Alteromonadaceae* family of the eubacterial kingdom (not shown).

*Ph*EF-Ts, like *Ec*EF-Ts, forms with the homologous EF-Tu a dimeric complex. In the three-dimensional structure of the *E. coli* complex an $(EF\text{-}Ts{\cdot}EF\text{-}Tu)_2$ organization was found, in which the EF-Ts component forms a tight dimer (*6*). This quaternary structure differs from that of the *T. thermophilus* complex (*8*), which showed instead an $EF\text{-}Ts_2{\cdot}2EF\text{-}Tu$ quaternary topology. A dimeric exchange factor was also shown in the case of the archaeal functional analogue EF-1β from *S. solfataricus* (*34*). The high sequence identity between *Ph*EF-Ts and *Ec*EF-Ts (Figure 7), and between *Ph*EF-Tu and *Ec*EF-Tu (86%) (*14*), led to the construction of a 3D model for *Ph*EF-Ts, based on the structural coordinates of the *E. coli* EF-Ts·EF-Tu complex (*6*). The model was almost superimposable on the template (not shown) except for two differences observed in the corresponding regions of *Ec*EF-Ts connecting helix 5 and helix 6 in the subdomain N, and strand 5 and helix 8 in the subdomain C. These two regions, in the model of *Ph*EF-Ts, were shorter because in the structural alignment (Figure 7) a gap in the *Ph*EF-Ts sequence at the level of residues Asp106 and Lys158 of *Ec*EF-Ts was introduced. In addition, all the amino acid residues involved in the dimerization domain of *Ec*EF-Ts were conserved, except two of them that were slightly different in *Ph*EF-Ts. These residues were Tyr177 and Glu197, which in *Ec*EF-Ts corresponded to Phe179 and Asp199, respectively. Also the consensus sequence TDFV, involved in the interaction with domain I of *Ec*EF-Tu, in *Ph*EF-Ts was well conserved (T79DFV); additional differences were found in the amino acid residues that in *Ec*EF-Ts were in contact with *Ec*EF-Tu. These were Ala5, Val234, and Met278, which in *Ph*EF-Ts corresponded to Thr5, Ile232, and Glu276. Therefore, it will be of interest to see if also the 3D structure of the *P. haloplanktis* EF-Ts·EF-Tu complex will have the same structural features as the *E. coli* complex.

At 0 °C *Ph*EF-Ts can promote an exchange reaction that is faster compared to that for the *S. solfataricus* system measured at 50 °C (*5*) but significantly slower than that reported for the *E. coli* system (*35*, *36*). The high concentration of *Ph*EF-Ts found in *P. haloplanctis* (around 6% of the total proteins) is in contrast to the low concentration of this nucleotide exchange factor found in the other eubacterial (*25*, *37*, *38*) or archaeal (*5*) systems; therefore, it can be speculated that the low efficiency in the nucleotide exchange on *Ph*EF-Tu, probably due to the low environmental temperature, is enhanced by a high content of the nucleotide exchange factor in the *P. haloplanktis* cells. This finding confirmed the hypothesis that the increase in protein concentration is one of the mechanisms developed by psychrophiles to allow metabolic rates at low temperatures compatible with those of mesophilic organisms at higher temperatures (*9*, *10*). Finally, it cannot be excluded that the high content of *Ph*EF-Ts in this cold-adapted microorganism might be related to other functions displayed by this protein; in fact, this feature was already found for *Ec*EF-Ts, which was reported to assist the folding of *Ec*EF-Tu, in particular conditions (*39*).

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