Psychrophilic Elongation Factor Tu from the Antarctic *Moraxella* sp. Tac II 25: Biochemical Characterization and Cloning of the Encoding Gene[†,‡]

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ABSTRACT: The elongation factor Tu was isolated from a psychrophilic eubacterial Antarctic *Moraxella* strain (*Mo*EF-Tu) and its molecular and functional properties were determined. It catalyzed the synthesis of poly(Phe) and bound specifically guanine nucleotides with an affinity for GDP about 12-fold higher than that for GTP. The affinity toward guanine nucleotides was lower than that of other eubacterial EF-Tu. The intrinsic GTPase activity of *Mo*EF-Tu was hardly detectable but was accelerated by 2 orders of magnitude in the presence of the antibiotic kirromycin (GTPase^k). Such a property resembled *Escherichia coli* EF-Tu (*Ec*EF-Tu) even though the affinity of *Mo*EF-Tu for the antibiotic was lower. *Mo*EF-Tu showed a thermophilicity higher than that of *Ec*EF-Tu; its temperature for half-denaturation was 44 °C. The *Mo*EF-Tu encoding gene corresponding to *E. coli tufA* was cloned and sequenced. The translated protein had a calculated molecular weight of 43 288 and contained the GTP-binding sequence motifs. Concerning its primary structure, *Mo*EF-Tu showed sequence identity with *E. coli* and *Thermus thermophilus* EF-Tu equal to 84% and 74%, respectively, while the identity with EF-1α from the archaeon *Sulfolobus solfataricus* was equal to 32%.

Extremophiles are organisms living under extreme environmental conditions such as very low or high pH (acidophiles and alkalophiles), high salt concentration (halophiles), high hydrostatic pressure (barophiles), and very high (thermophiles and hyperthermophiles) or low (psychrophiles) temperatures (*1*). Adaptation of proteins to such extreme physical conditions and the effect of temperature on their stability and catalytic properties have been investigated in several cases, showing that the temperature optimum of enzymes represents a compromise between the rate of the catalyzed reaction and their thermal inactivation (*2, 3*). Therefore, activity and stability are negatively correlated: at high temperatures thermophilic enzymes are more stable and less catalytically efficient, whereas at low temperatures mesophilic enzymes are less thermostable but more active (*3*). Recently, the attention of several investigators has been devoted to cold enzymes isolated from psychrophilic organisms. These enzymes are characterized by high catalytic efficiency at temperatures in the range 4–20 °C, at which mesophilic or thermophilic enzymes display low activity or no activity at all. In addition, they possess the ability to undergo conformational changes that favor the interaction with the substrate, thus compensating their slow catalytic

rates elicited at low temperatures. On the other hand, such flexibility is also responsible for the thermal instability that is a common property of the cold proteins (*4, 5*). Therefore, cold enzymes represent an important class of proteins in which the structure–function relationships can be investigated, especially if such properties are compared with those of homologous mesophilic and thermophilic counterparts. The elongation factor Tu (EF-Tu[1] in bacteria; EF-1α in eucarya and archaea) is a suitable molecule for such investigations since it is a ubiquitous enzyme and its functional properties and structural features have been extensively studied in a wide variety of mesophilic and thermophilic organisms (*6–8*). EF-Tu catalyzes the binding of the aminoacyl tRNA to the A site of the ribosome; it interacts with nucleotides, ribosome-mRNA, several other proteins and it also displays a GTPase activity. In addition to its role in translation, EF-Tu constitutes one of the subunits of the Qβ replicase, it acts as a chaperone in the renaturation of urea-denatured rhodanese, and it displays a disulfide isomerase activity (for references see refs *9* and *10*). Therefore EF-Tu is a multifunctional protein, and in investigations related to function–structure relationships its

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[1] Abbreviations: EF, elongation factor; *Mo*, *Moraxella*; *Ec*, *Escherichia coli*; *Ss*, *Sulfolobus solfataricus*; *Tt*, *Thermus thermophilus*; *Ta*, *Thermus aquaticus*; PAGE, polyacrylamide gel electrophoresis; k_{+1}, association rate constant; k_{-1}, dissociation rate constant; K_{act}, concentration of kirromycin that half-maximally activates the guanosine triphosphatase of elongation factor Tu; $T_{1/2}$, temperature for half heat inactivation or half thermal denaturation; GTPase^k, elongation factor Tu guanosine triphosphatase activity measured in the presence of kirromycin; EF-Tu$_{free}$, elongation factor Tu freed from the nucleotide; HPLC, high-pressure liquid chromatography; PCR, polymerase chain reaction.

various individual activities can be used as tools to evaluate the effects produced by the living environment or by engineered modifications. Finally, the amino acid sequences of EF-Tu/EF-1α have been used for phylogenetic relationships in the universal tree (*11, 12*). The molecular, physical and biochemical properties of the EF-1α isolated from the hyperthermophilic archaeon *Sulfolobus solfataricus* (optimum temperature for growth 87 °C, pH 3.5) have been thoroughly investigated (*13−15*). This work now describes the purification and characterization of a psychrophilic EF-Tu isolated from an Antarctic strain of *Moraxella* (growth temperature 4−20 °C) and the cloning of its encoding gene. Its physical, functional, and structural properties are compared with those of mesophilic and thermophilic EF-Tus.

MATERIALS AND METHODS

Chemicals, Enzymes, and Buffers. Restriction enzymes, modifying enzymes, labeled compounds, and chemicals were as already reported (*16*); plasmid DNA, genomic DNA, and labeled probes were prepared as described (*17*). GDP, GTP, phospho*enol*pyruvate, pyruvate kinase, and myokinase were purchased from Sigma. The chromatographic media were from Pharmacia Biotech.

The following buffers were used: buffer A, 20 mM Tris· HCl, pH 7.8, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, and 10% (v/v) glycerol; buffer B, 20 mM Tris·HCl, pH 7.8, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, and 50 mM KCl; buffer C, 50 mM imidazole·acetate, pH 7.5, 10 mM $MgCl_2$, 200 mM NH_4Cl, and 1 mM dithiothreitol. Prior to their use, GTP, [^{3}H]GTP, and [γ-^{32}P]GTP were incubated for 20 min at 25 °C in buffer B in the presence of 50−500 μM phospho*enol*pyruvate and 4−40 μg of pyruvate kinase.

Enzyme Assays. *Mo*EF-Tu and *Ec*EF-Tu were assayed by their ability to form a binary complex with [^{3}H]GDP (*18*). The reaction mixture contained 0.3−1.0 μM *Mo*EF-Tu and 3−10 μM [^{3}H]GDP (specific activity 100−200 cpm/pmol) in buffer B; after incubation for 30 min at 15 °C to reach the equilibrium, an aliquot was transferred onto a nitrocellulose filter, which was then washed twice with buffer B. The filters were dried and counted for radioactivity.

The GTPase activity of *Mo*EF-Tu was assayed in buffer C by the molybdate/isopropyl acetate method as described (*14*). The reaction mixture contained 0.1−0.3 μM *Mo*EF-Tu, 5−10 μM [γ-^{32}P]GTP (specific activity 1500−2000 cpm/ pmol), and 50 μM kirromycin; the reaction was followed kinetically, and at appropriate time intervals aliquots were withdrawn and analyzed for the ^{32}P$_i$ released. Blanks run in the absence of the enzyme, which never exceeded 5% of the activity determined in the presence of EF-Tu, were subtracted. K_m for GTP and k_{cat} were obtained from the measurements of the reaction rate at different [γ-^{32}P]GTP concentration and were derived from Lineweaver−Burk plots.

Purification of MoEF-Tu. Antarctic *Moraxella* sp. TAC II 25 cells were grown on LB medium at 4 °C and collected after 4 days of incubation during the exponential phase of growth. Wet cells (33 g) were suspended in 70 mL of buffer A and disrupted by two French press strokes at about 5 MPa in the presence of 200 μM phenylmethanesulfonyl fluoride and 50 μg/mL trypsin inhibitor. Cellular debris was removed by centrifugation at 30000*g* and the cell-free extract (S-100)

was separated from the ribosomal pellet by ultracentrifugation at 100000*g*. All steps were carried out at 4 °C. 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 buffer A containing 100 mM KCl, a linear 100−300 mM KCl gradient in buffer A (2.2 L total volume) was applied. The fractions revealing *Mo*EF-Tu activity eluted at about 200 mM KCl and were pooled together, dialyzed against buffer A, and then loaded onto a Mono Q HR 10/10 equilibrated at 3 mL/min with buffer A. The bound proteins were eluted with a linear 0−400 mM KCl gradient in buffer A. Fractions containing *Mo*EF-Tu 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 containing 10% glycerol. The fractions containing *Mo*EF-Tu 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 10 μM GDP, and stored at −20 °C. Under these conditions *Mo*EF-Tu·GDP was stable for at least 6 months.

Preparation of Nucleotide-Free MoEF-Tu and EcEF-Tu. *Mo*EF-Tu and *Ec*EF-Tu freed from the bound nucleotide were prepared by the small gel-filtration column method (*18*). The concentration of EF-Tu$_{free}$ was determined by titration with [^{3}H]GDP (see above). Preformed binary complexes were prepared by incubating 1.5 μM EF-Tu$_{free}$ with 3 μM GDP, GTP, [^{3}H]GDP, or [^{3}H]GTP (specific activity 2000−4000 cpm/pmol) in buffer B for 30 min at 15 °C.

Determination of the Association and Dissociation Rate Constants and Equilibrium Dissociation Constant of MoEF-Tu·GDP, MoEF-Tu·GTP, and MoEF-Tu·Kirromycin. The association rate constant of *Mo*EF-Tu·[^{3}H]GDP and *Mo*EF-Tu·[^{3}H]GTP was determined by incubating 0.3 μM *Mo*EF-Tu$_{free}$ and 0.3 μM [^{3}H]GDP (specific activity 5922 cpm/pmol) or [^{3}H]GTP (specific activity 5706 cpm/pmol) in 480 μL final volume of buffer B as described (*18*). The determination of the dissociation rate constant of *Mo*EF-Tu·[^{3}H]GDP or *Mo*EF-Tu·[^{3}H]GTP or *Ec*EF-Tu·[^{3}H]GDP was carried out by incubating 0.27 μM preformed radioactive binary complex in 250 μL final volume of buffer B; the dissociation reaction was started by adding a 1000-fold molar excess of unlabeled GDP or GTP and followed kinetically (*18*). The apparent equilibrium dissociation constant K_d' of the *Mo*EF-Tu· nucleotide·kirromycin complex was derived by native gel-shift experiments (*19*).

Evaluation of Thermal Stability of MoEF-Tu. The heat stability of *Mo*EF-Tu was investigated by incubating 4 μM protein in buffer B for 10 min at different temperatures in the interval 40−60 °C in the absence or in the presence of 50 μM kirromycin. After the heat treatment the mixtures were cooled on ice for at least 30 min and then assayed for their GDP binding ability and the residual GTPase[k]. The heat denaturation was evaluated by the melting curves obtained by measuring the difference $A_{286} - A_{274}$ (*16, 20*) in the temperature range 10−60 °C.

Cloning of the MoEF-Tu Encoding Gene. A *Moraxella* DNA library was prepared in a pUC18 cloning vector by ligation after both DNAs were digested with *Pst*I restriction enzyme. After transformation of *E. coli* TG1 cells, about 2 × 10^3 colonies were analyzed by using as probe the ^{32}P-labeled degenerate oligonucleotide AA(AG)AA(AT)AT-

GAT(AT)AC(AT)GG(AT)GC (Mo) derived from the amino acid sequence of the heptapeptide KNMITGA conserved in all the EF-1α/EF-Tu (*21*). PCR on *Mo*DNA was performed, with the forward primer GA(CT)ACiCCiGGiCA(CT)GTi-GA(CT)TT (Mo7) deduced from the amino acid sequence D[88]TPGHVDF of *Ec*EF-G (*22*), whereas the reverse primer ACGATGTTTGCAACAAC (Mo9) was derived from the sequence of the 3′ flanking region of the *Mo*EF-Tu gene. Hybridization was performed as already described (*23*).

Other Methods. SDS−PAGE was carried out on a 12% polyacrylamide gel according to the method of Laemmli (*24*). Native gel electrophoresis was performed on a polyacrylamide gel prepared in 375 mM Tris·HCl buffer, pH 8.8. The gel was run in 25 mM Tris−glycine buffer, pH 8.3, containing 20 μM GDP or GTP in the upper chamber. Protein was determined by the method of Bradford (*25*). The amino acid sequence of a *Mo*EF-Tu internal tryptic peptide was obtained after digestion of purified *Mo*EF-Tu by incubating 1.35 mg/mL protein for 16 h at 37 °C in buffer B in the presence of 8 μg/mL tosylphenylalanine chloromethyl ketone-treated trypsin. The hydrolytic reaction was stopped by adding 66 μg/mL soybean trypsin inhibitor. Peptides were separated by HPLC (Varian) on a C18 (Vydac) column as described (*26, 27*). The amino acid sequence was determined by automated Edman degradation (*26*). Electrospray-mass spectrometry (ES/MS) experiments for molecular weight determination were performed as described (*27*). Nucleotide sequencing of the *Mo*EF-Tu gene was performed on both DNA strands by using the T7 sequencing kit (Promega) and appropriate primers. Southern and Northern blots were performed as described (*17*). The three-dimensional modeling of *Mo*EF-Tu was carried out with the help of the SwissModel server (*28, 29*) and the crystal structure coordinates of EF-Tu·GDP from *T. aquaticus* (*30*), *E. coli* (*31*), and bovine mitochondria (*32*) were used as templates (PDB entries 1TUI, 1EFC, and 1D2E, respectively).

RESULTS

*Molecular Properties of MoEF-Tu. Mo*EF-Tu was purified from the ribosomal cell-free extract of Antarctic *Moraxella* sp. TAC II 25 with a total recovery of about 24% and an enrichment of the ability to bind GDP of about 42-fold as compared to the S-100 fraction. *Mo*EF-Tu is a monomeric protein of 43 288 Da as determined by ES/MS. A slightly higher value of 47 500 Da was estimated by both gel filtration at neutral pH and SDS−PAGE.

*MoEF-Tu Catalyzes the Synthesis of Poly(Phe). Mo*EF-Tu was also identified for its ability to support the synthesis of poly(Phe). In fact, an *E. coli in vitro* system containing all the components required for poly(Phe) synthesis except *Ec*EF-Tu was unable to promote phenylalanine polymerization. The addition to such a system of purified *Mo*EF-Tu promoted the synthesis of poly(Phe) at a rate that at 15 °C was about 1.3-fold slower compared to *Ec*EF-Tu (Figure 1).

MoEF-Tu Binds Specifically Guanine Nucleotides. At 15 °C *Mo*EF-Tu was able to bind [³H]GDP (Figure 2). Competitive binding experiments showed that [³H]GDP was displaced by GTP but not by ATP, thus demonstrating that *Mo*EF-Tu bound specifically guanine nucleotides. The data of the titration of *Mo*EF-Tu with [³H]GDP analyzed according to the Scatchard equation indicated one nucleotide
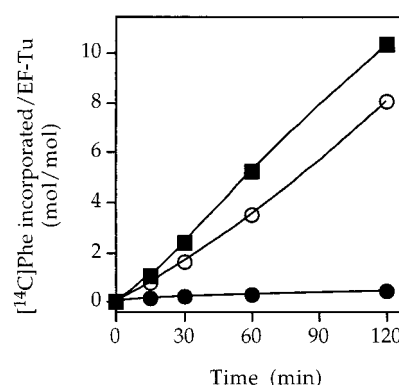

FIGURE 1: Poly(U)-directed poly(Phe) synthesis supported by *Mo*EF-Tu. The reaction mixture (150 μL) contained 50 mM Hepes·KOH buffer, pH 7.5, 70 mM NH$_4$Cl, 10 mM MgCl$_2$, 7 mM β-mercaptoethanol, 0.9 mM ATP, 1.0 mM GTP, 3.8 mM phospho*enol*pyruvate, 30 μg/mL pyruvate kinase, 2 units/mL myokinase, 50 nM *Ec*EF-Ts, 0.8 μM *Ec*EF-G, 20 μM [¹⁴C]Phe (specific activity 450 dpm/pmol), 3 μM *Ec*tRNA^{Phe}, 26 nM Phe-tRNA synthetase, 1.5 μM *E. coli* ribosome with tRNA^{Phe} preloaded at the P-site, 100 μg/mL poly(U), in the absence (●) or in the presence of 0.5 μM *Ec*EF-Tu (■) or *Mo*EF-Tu (○). The final mixture was incubated at 15 °C, and 20 μL aliquots were withdrawn at the indicated times and analyzed for poly(Phe) synthesized.
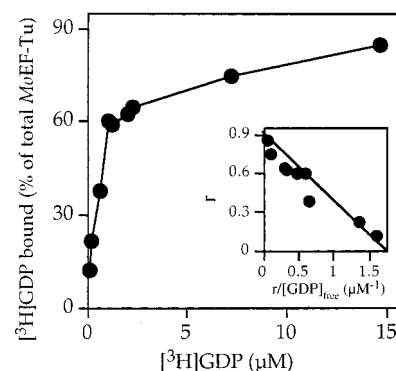

FIGURE 2: Binding of [³H]GDP to *Mo*EF-Tu. The titration of *Mo*EF-Tu with [³H]GDP was carried out in 100 μL final volume of buffer B containing 0.4 μM *Mo*EF-Tu and [³H]GDP (specific activity 2550 cpm/pmol) at the indicated concentration. The reaction mixture was incubated for 30 min at 15 °C, and the amount of the formed *Mo*EF-Tu·[³H]GDP complex was determined in triplicate on 35 μL aliquots as described under Materials and Methods. Inset: Scatchard plot of the data; *r* corresponds to the moles of [³H]GDP bound per mole of *Mo*EF-Tu.

binding site on *Mo*EF-Tu (Figure 2, inset). At 15 °C the values of k_{-1} and k_{+1} were 0.018 min^{-1} and 0.057 min^{-1}·μM^{-1}, respectively; from these data a value of 0.32 μM was calculated for the K_d of *Mo*EF-Tu·[³H]GDP complex (Table 1), a value very similar to that derived from the Scatchard plot (0.43 μM) reported in the inset to Figure 2. This value was 58- and 166-fold higher than those found for *Ec*EF-Tu·[³H]GDP at 30 °C (*18*) and *Tt*EF-Tu·[³H]GDP at 15 °C (*33*), respectively; such a low affinity toward GDP has recently been found also for bovine mitochondrial EF-Tu (*32, 34*). It is remarkable that the on rate of the [³H]-GDP binding for *Mo*EF-Tu was 800-fold slower than that elicited by *Ec*EF-Tu. The affinity at 15 °C of *Mo*EF-Tu for [³H]GTP was determined by using the same experimental approach as for [³H]GDP. The values of k_{-1} and k_{+1} were 0.23 min^{-1} and 0.058 min^{-1}·μM^{-1}, respectively, which accounted for a value of 4 μM for the K_d of the *Mo*EF-Tu·[³H]GTP complex. This value was about 7- and 137-fold

Table 1: Affinity of *Mo*EF-Tu for Guanine Nucleotides: Comparison with Mesophilic and Thermophilic Eubacterial *Ec*EF-Tu and *Tt*EF-Tu and Hyperthermophilic Archaeal *Ss*EF-1α

	temp (°C)	k_{-1} (min^{-1})	k_{+1} (min^{-1}·μM^{-1})	K_d (μM)	ref
*Mo*EF-Tu·GDP	15	0.018 ± 0.001^a	0.057 ± 0.002^a	0.32^b	this work
*Ec*EF-Tu·GDP	30	0.26	48	0.0055^b	18
*Tt*EF-Tu·GDP	15	0.15	83.3	0.0018^b	33
*Ss*EF-1α·GDP	60	0.14	0.09	1.5^c	35
*Mo*EF-Tu·GTP	15	0.23 ± 0.06^a	0.058 ± 0.004^a	4.0^b	this work
*Ec*EF-Tu·GTP	30	1.1	1.9	0.6^b	18
*Tt*EF-Tu·GTP	15			0.029^b	33
*Ss*EF-1α·GTP	60			35^d	35

^{a} Average of six determinations. ^{b} $K_d = k_{-1}/k_{+1}$. ^{c} Determined by Scatchard plot. ^{d} Determined by competitive binding experiments.

higher than those reported for *Ec*EF-Tu·[^{3}H]GTP at 30 °C (*18*) and *Tt*EF-Tu·[^{3}H]GTP at 15 °C (*33*), respectively. As evaluated from the K_d values summarized in Table 1, the highest affinity for GDP and GTP was displayed by *Tt*EF-Tu, followed by *Ec*EF-Tu, *Mo*EF-Tu, and *Ss*EF-1α (*35*) in that order. In all cases the affinity for GDP was higher than that for GTP, decreasing from 109-fold for *Ec*EF-Tu to 12-, 16-, and 23-fold for *Mo*EF-Tu, *Tt*EF-Tu, and *Ss*EF-1α, respectively. Therefore, the affinity for guanine nucleotides increased going from hyperthermophilic *Ss*EF-1α to psychrophilic, mesophilic, and thermophilic eubacterial EF-Tus.

GTPase Activity of MoEF-Tu. *Mo*EF-Tu displayed an intrinsic GTPase whose rate was measured as low as 1.2×10^{-4} mol of [γ-^{32}P]GTP hydrolyzed (mol of EF-Tu)$^{-1}$ min^{-1}. This was accelerated up to about 2 orders of magnitude by the antibiotic kirromycin, a property resembling that of *Ec*EF-Tu (*18, 36*). The extent of stimulation depended on the concentration of the antibiotic (Figure 3A). The highest effect was reached at 5 μM kirromycin, and it remained unchanged up to 50 μM. The enzymatic activity was specific for GTP, since the addition of ATP at a 10-fold higher concentration than GTP did not affect the rate of GTP hydrolysis (not shown). Compared to *Ec*EF-Tu, the GTPase^k of *Mo*EF-Tu showed the same pattern of dependence on the antibiotic but the maximum activity was about 2 times lower (Figure 3A). Such a lower sensitivity of *Mo*EF-Tu to the antibiotic was confirmed by the concentration of kirromycin required for half-maximal activation of its GTPase (K_{act}), corresponding to nearly twice that of *Ec*EF-Tu (Figure 3B). The kinetic parameters of GTPase^k of *Mo*EF-Tu are shown in Table 2 and compared with those determined under identical experimental conditions for the GTPase^k of *Ec*EF-Tu. They were all indicative of a weaker interaction with kirromycin of *Mo*EF-Tu as compared to *Ec*EF-Tu.

Binding of Kirromycin to MoEF-Tu·GTP and MoEF-Tu·GDP. The affinity of kirromycin for *Mo*EF-Tu was evaluated by experiments on PAGE under nondenaturing conditions (*19*). The minimum concentration of kirromycin required to observe the interaction depended on whether *Mo*EF-Tu was in the GTP (Figure 4A) or in the GDP (Figure 4B) bound form. In fact, the amount of kirromycin required to convert *Mo*EF-Tu·GTP into *Mo*EF-Tu·GTP·kirromycin was about 2–3 times lower than that needed to bind the antibiotic to *Mo*EF-Tu·GDP. From the electrophoretic patterns, the apparent equilibrium dissociation constant, K_d', at 0 °C derived
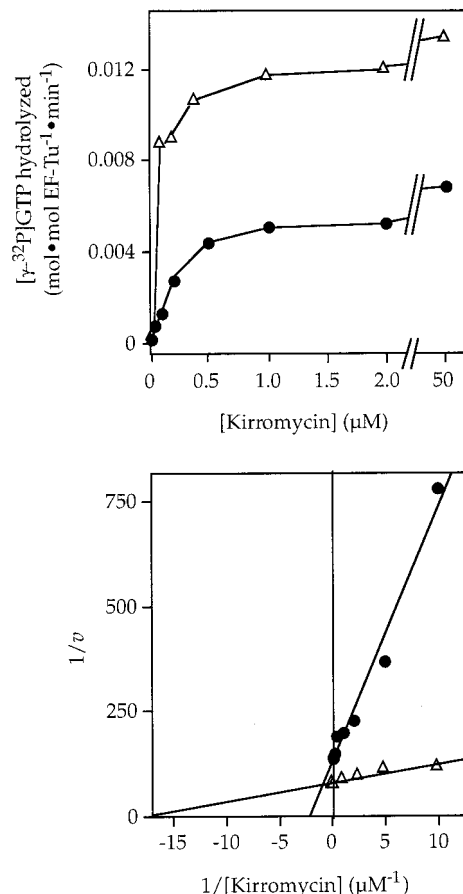

FIGURE 3: Effect of kirromycin on the intrinsic GTPase of *Mo*EF-Tu and *Ec*EF-Tu. (A) The reaction mixture contained 10 μM [γ-^{32}P]-GTP (specific activity 5760 cpm/pmol) 0.2 μM *Mo*EF-Tu (●) or *Ec*EF-Tu (△) and the indicated amounts of kirromycin in 200 μL final volume of buffer C. The reaction was carried out at 15 °C, and the amount of [γ-^{32}P]GTP hydrolyzed was followed kinetically for each concentration of the antibiotic as described under Materials and Methods. (B) Lineweaver−Burk plots of the data reported in panel A; the activity determined in the absence of kirromycin was subtracted.

for *Mo*EF-Tu·GTP and *Mo*EF-Tu·GDP complexed with kirromycin were 5.1 μM (average of six determinations) and 16 μM (average of three determinations), respectively. The higher value of K_d' derived from PAGE experiments, compared to K_{act} reported in Table 2, can be ascribed to the different methods used for the determination of the affinity of *Mo*EF-Tu for kirromycin.

Thermophilicity of MoEF-Tu. Kirromycin enhances the rate of the [^{3}H]GDP/GDP exchange on *Mo*EF-Tu·[^{3}H]GDP. In fact, at 15 °C the rate of the exchange reaction was 0.015 min^{-1} and 0.44 min^{-1} in the absence or in the presence of 50 μM kirromycin, respectively.

To evaluate the thermophilicity of *Mo*EF-Tu the [^{3}H]GDP/GDP exchange rate was measured in the temperature range 0−60 °C. This rate increased about 330-fold in the temperature range 4−55 °C. Above this latter temperature inactivation occurred (Figure 5). Vice versa, under identical experimental conditions *Ec*EF-Tu showed at each temperature a higher value of k_{-1} and the maximum was reached at 60 °C, after which inactivation occurred. However, the rate of nucleotide exchange increased by about 100 times in the temperature range 4−55 °C. The energy of activation was 85 and 71 kJ·mol^{-1} for *Mo*EF-Tu and *Ec*EF-Tu, respectively.

Table 2: Kinetic Parameters of the Kirromycin-Induced *Mo*EF-Tu and *Ec*EF-Tu GTPase[a]

	k_{cat} (min^{-1})	$K_{m(GTP)}$ (μM)	k_{cat}/K_m (min$^{-1} \cdot \mu$M^{-1})	$K_{i(GDP)}$ (μM)	K_{act}(kirromycin) (μM)
*Mo*EF-Tu	0.007 ± 0.003	0.36 ± 0.06	0.019	0.16 ± 0.03	0.44 ± 0.05
*Ec*EF-Tu	0.013 ± 0.003	0.13 ± 0.03	0.1	0.09 ± 0.02	0.10 ± 0.02

[a] Values represent the average of 3−7 determinations carried out at 15 °C.
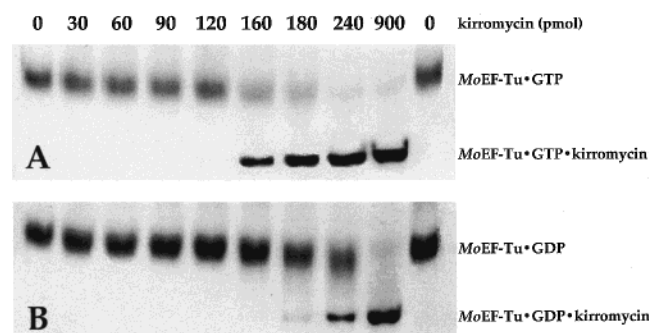
FIGURE 4: Binding of kirromycin to *Mo*EF-Tu. A 40-μL final volume of buffer C contained 40 pmol of *Mo*EF-Tu·GTP (gel A) or *Mo*EF-Tu·GDP (gel B) and kirromycin at the indicated amounts (picomoles). The reaction mixtures were incubated for 10 min at 0 °C and then loaded on a native 11% polyacrylamide gel. For other details see Materials and Methods.
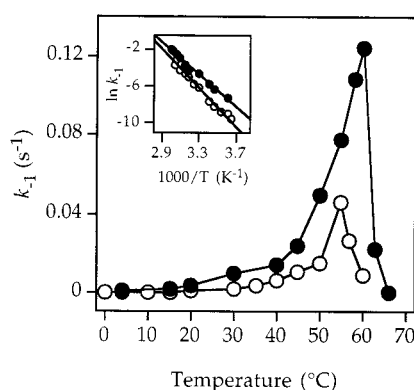
FIGURE 5: Effect of temperature on the [³H]GDP/GDP exchange on the *Mo*EF-Tu·[³H]GDP complex. A 300-μL final volume of buffer B contained 0.6 μM *Mo*EF-Tu·[³H]GDP (○) or *Ec*EF-Tu·[³H]GDP (●). The dissociation was started by adding GDP up to 600 μM final concentration and was followed kinetically by determining the radiolabeled binary complex at the various temperatures. Inset: Arrhenius plot of the data in the range 0−55 °C for *Mo*EF-Tu and 5−60 °C for *Ec*EF-Tu.

In the case of *Mo*EF-Tu, the energy of activation of the exchange reaction in the presence of kirromycin remained 85 kJ·mol^{-1}. For *Ec*EF-Tu the temperature effect in the presence of the antibiotic could not be evaluated because the nucleotide exchange rate on *Ec*EF-Tu at high temperature was too fast to be measured.

The thermophilicity of *Mo*EF-Tu was also evaluated by measuring the k_{cat} of its GTPase[k] at increasing temperatures (Figure 6). The Arrhenius plot showed a break point at about 22 °C (Figure 6, inset), thus indicating that at this temperature a conformational change on *Mo*EF-Tu might occur. However, a shift in the temperature dependence of a rate-limiting step in the activation reaction could not be excluded. It is worth noting that such a break was not observed when the *Mo*EF-Tu·[³H]GDP dissociation rate in the presence of kirromycin was analyzed.
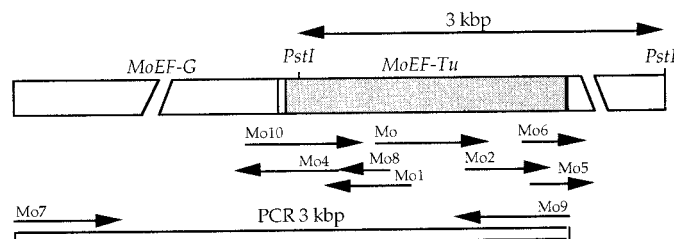
Thermostability of MoEF-Tu. The effect of temperature on the stability of *Mo*EF-Tu·GDP was evaluated either by
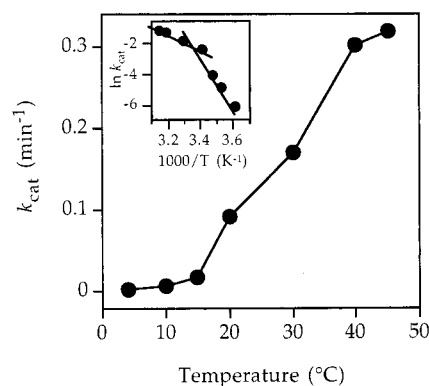
FIGURE 6: Effect of the temperature on the k_{cat} of GTPase[k] of *Mo*EF-Tu. A 200-μL final volume of buffer C contained 0.2 μM *Mo*EF-Tu and 0.05−0.6 μM [γ-³²P]GTP (specific activity 35 760 cpm/pmol). At each temperature the hydrolytic reaction was followed kinetically by measuring the amount of the ³²P$_i$ released at appropriate time intervals depending on the temperature. The k_{cat} values were determined by Lineweaver−Burk plots. Inset: Arrhenius plot of the data.
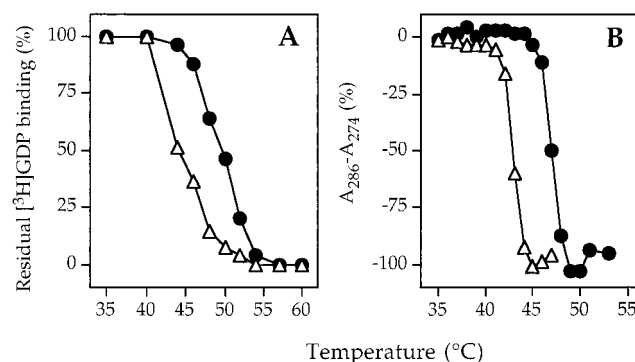
FIGURE 7: Heat inactivation and thermal denaturation of *Mo*EF-Tu: effect of kirromycin. Residual [³H]GDP binding ability (panel A) or UV melting curves (panel B) of *Mo*EF-Tu·GDP treated at the indicated temperatures in the absence (△) or in the presence (●) of 50 μM kirromycin are shown. For other details see Materials and Methods.

measuring the [³H]GDP/GDP exchange ability (Figure 7A) or by examining the UV melting curves of *Mo*EF-Tu·GDP (Figure 7B). *Mo*EF-Tu·GDP was half-inactivated after 10 min of treatment at 44 °C, a temperature 40 °C higher than that of the growth temperature of *Moraxella*. Similar results were obtained with the GTPase[k] of *Mo*EF-Tu. For the mesophilic *Ec*EF-Tu·GDP and the hyperthermophilic *Ss*EF-1α, such a difference was equal to 14 and 7 °C, respectively (*16*). Almost identical results were derived from the analysis of the UV melting curves (Figure 7B). The addition of 50 μM kirromycin raised the $T_{1/2}$ of *Mo*EF-Tu by 5−6 °C in the case where heat inactivation or thermal denaturation was performed (Figure 7).

MoEF-Tu Encoding Genes. Southern blot analysis indicated that two highly homologous *Mo*EF-Tu genes are present in the genomic *Moraxella* DNA (not shown). The screening of the *Moraxella* DNA library performed as

FIGURE 8: Sequence of the *Mo*EF-Tu gene. (A) Sequencing strategy. Arrows indicate sequence obtained by use of the corresponding primers. (B) Nucleotide sequence of the *Mo*EF-Tu gene and deduced amino acid sequence. Putative ribosomal binding site is shown in italic type. Potential promoter element, palindromic sequence, and transcription termination signal are underlined. The amino acid sequence determined on a tryptic peptide of the protein is also underlined. The nucleotide sequence and the deduced amino acid sequence of the C-terminal part of *Mo*EF-G is also reported (nucleotides −119 to −63).

described under Materials and Methods led to the isolation of a recombinant clone containing a 3 kbp fragment, which was sequenced. This clone did not contain the entire *Mo*EF-Tu gene because a *Pst*I restriction site was present in the 5′-coding region of the *Mo*EF-Tu gene. To obtain the complete coding sequence a PCR was performed, with

Moraxella DNA as template and the oligonucleotides Mo7 and Mo9 as primers (see Materials and Methods). The synthesized product, about 3 kbp, was cloned into the pGEM T-easy vector and sequenced according to the strategy reported in Figure 8A. Figure 8B showed the nucleotide sequence of the *Mo*EF-Tu gene. The 5′-flanking region was

62 nucleotides long and contained a 2-fold symmetry segment positioned at −32 to −49, as numbered from the *Mo*EF-Tu start codon, and a potential ribosomal binding site (*37, 38*). Furthermore, a possible binding site of RNA polymerase at positions −65 to −70 and −95 to −100 was also present (*39*). In the 5′ region, a partial coding segment homologous to the C-terminal region of eubacterial EF-G was also contained. This finding indicated that the sequenced *Mo*EF-Tu gene corresponded to the *E. coli tufA* gene. The 3′-flanking region contained a possible trascription termination signal. It was found that synonymous codons most frequently used in *Mo*EF-Tu gene were those ending in A and T. For instance, among the 39 Val residues, 19 are encoded by GTA, 19 by GTT, and 1 by GTG.

To detect the size of the transcripts containing the *Mo*EF-Tu genes, total RNA from *Moraxella* was analyzed by Northern blot. Only a major transcript was detected corresponding to an RNA of about 1.3 kb (not shown). The *Mo*EF-Tu gene encoded a protein of 393 amino acid residues including the initial methionine.

The 3D model of *Mo*EF-Tu, obtained with *Ta*EF-Tu·GDP (*30*) *Ec*EF-Tu·GDP (*31*), and bovine mitochondrial EF-Tu·GDP (*32*) as templates, was almost superimposable to the templates (not shown). Only small differences were observed in the catalytic domain (G-domain) of *Mo*EF-Tu. The multiple alignment derived from the modeling is reported in Figure 9. The amino acid identity with *Mo*EF-Tu was 73%, 86%, and 49% for *Ta*EF-Tu, *Ec*EF-Tu, and bovine mitochondrial EF-Tu, respectively. The overall identity among these four sequences was 48%.

DISCUSSION

This is the first report of an EF-Tu isolated from a psychrophilc eubacterium. The content of EF-Tu in the *Moraxella* sp. TAC II 25 cells was estimated to be approximately 3% of the soluble proteins, a concentration lower than that reported for other microorganisms (*13, 40*). As all the GTP binding proteins, *Mo*EF-Tu elicited an intrinsic turnover GTPase activity that, as in the case of *Ec*EF-Tu, was strongly stimulated by the antibiotic kirromycin. Compared to GTPase^k of *Ec*EF-Tu, the K_m for GTP of *Mo*EF-Tu was almost 3 times higher; the catalytic efficiency was 5 times lower as a consequence of the fact that k_{cat} of GTPase^k of *Mo*EF-Tu was 50% lower. This feature was in contrast with the general rule according to which, at any temperature, psychrophilic enzymes display a higher catalytic efficiency compared to their mesophilic or thermophilic counterparts (*41, 42*). The explanation for this finding may reside in the lower affinity of *Mo*EF-Tu for kirromycin compared to *Ec*EF-Tu (Figure 3 and Table 2). In *Ec*EF-Tu the binding site for the antibiotic is very likely located at the interface between the EF-Tu domains I and III (*43, 44*) and involves L120, Q124, Y160, G316, Q329, K357, A375, and E378. All these residues are conserved in *Mo*EF-Tu (Figure 9), and therefore its lower affinity for kirromycin probably resides in some differences in the local structural requirements. Regarding the effects of kirromycin on the affinity of *Mo*EF-Tu for nucleotides, the antibiotic provoked a 2-fold increase in the affinity of *Mo*EF-Tu for GDP [cf. $K_{I(GDP)}$ in Table 2 with $K_{d(GDP)}$ in Table 1]. The effect of the antibiotic was much greater on the affinity of *Mo*EF-Tu for GTP, since in

FIGURE 9: Alignment of the amino acid sequence of *Mo*EF-Tu (Mo) with that of *Ec*EF-Tu (Ec), *Ta*EF-Tu (Ta), and bovine mitochondrial EF-Tu (Mi). Asterisks and dots indicate sequence identities and similarities, respectively. Residues shown in italic type refer to the consensus sequences for GTP binding (*51*); residues highlighted in gray are those putatively involved for kirromycin binding (*43, 44*); residues highlighted in black represent the sequence elements involved in the regulation of the affinity of EF-Tu toward guanine nucleotides.

the presence of kirromycin the affinity for the nucleotide became 10-fold higher than in its absence [cf. $K_{d(GTP)}$ in Table 1 with $K_{m(GTP)}$ in Table 2].

*Mo*EF-Tu displays an affinity for guanine nucleotides lower than that elicited by other eubacterial EF-Tu, whose K_d values fall in the nanomolar range (*18, 33*), but similar to that shown by bovine mitochondrial EF-Tu (*32*) and eucaryal (*45*) or archaeal (*13*) EF-1α, for which the dissociation constant is in the micromolar range. It has been recently proposed (*32*) that some of the reasons for the higher affinity of *Ec*EF-Tu toward GDP and GTP (*18, 33*) are a rigid conformation of the region M$_{139}$VDDEEL, the presence of a salt bridge between R121 and the conserved E150, and the presence of the bulky side chain of W183. In mitochondrial EF-Tu the MVDDEEL sequence is not conserved, the salt bridge is not present because arginine is substituted by valine, and the bulky side chain of tryptophan is substituted by the smaller side chain of leucine (*32*). Concerning *Mo*EF-Tu, only the absence of the salt bridge seems to be relevant,

because a glutamine (Q168) is found in place of the arginine, whereas the other two sequence elements are conserved (Figure 9).

The thermophilicity of *Mo*EF-Tu was demonstrated by the increase in the rate of the [³H]GDP/GDP exchange on *Mo*EF-Tu·[³H]GDP in the temperature range of 0−55 °C (Figure 5). Compared to *Ec*EF-Tu, the temperature for maximum nucleotide exchange activity of *Mo*EF-Tu was only 5 °C lower, despite the 33 °C difference in the growth temperature of the two microorganisms. The addition of kirromycin increased notably the rate of nucleotide exchange on *Mo*EF-Tu·GDP but it did not have any effect on the energy of activation of the reaction. This suggested that the stimulatory effect of the antibiotic was not due to a different conformation of the activated state but rather to a stimulation of the exchange rate only. Different was the case when the thermophilicity was evaluated on the basis of the k_{cat} of GTPase^k of *Mo*EF-Tu, since the Arrhenius plot showed a break at 22 °C (Figure 6). Since such a break was observed only with *Mo*EF-Tu·GTP·kirromycin, it can be inferred that a conformational change provoked on *Mo*EF-Tu by the antibiotic might consist in the alteration of structural interactions associated to the hydrolysis of GTP.

The observation that the heat inactivation (Figure 7A) and thermal denaturation curves (Figure 7B) showed identical profiles indicated that structural modifications of *Mo*EF-Tu provoked by thermal treatment involved the whole molecule and not the catalytic domain only. A remarkable observation was that the temperature for half-inactivation of *Mo*EF-Tu was about 40 °C higher than the growth temperature of *Moraxella*. This difference was much greater than that of 14 °C found for the mesophilic EF-Tu from *E. coli* and that of 7 °C measured for *Ss*EF-1α (*16*). Therefore, if the thermal stability is evaluated by the difference between the growth temperature of the source organism and the temperature for half-inactivation of *Mo*EF-Tu/*Ec*EF-Tu/*Ss*EF-1α, the conclusion is that *Mo*EF-Tu is much more stable than its mesophilic and thermophilic counterparts even though its intrinsic stability is reduced. We like to note that, like *Ec*EF-Tu, *Mo*EF-Tu contains a phenylalanine (Phe47) in the homologous position of Tyr48 in *Tt*EF-Tu·GppNHp. Tyr48 is thought to enhance the thermostability by forming an additional hydrogen bond on the α-phosphate of the nucleotide (*46*).

Like *E. coli* EF-Tu, *Moraxella* EF-Tu also appears to be encoded by two genes. The *Mo*EF-Tu gene isolated in this work corresponded to the *E. coli tufA* gene because it is contained in a genomic region similar to that of the *E. coli str* operon (*47*). Differently from *Ec*EF-Tu (*47*), *Mo*EF-Tu is transcribed as a single unit as suggested by the size of the transcript. This finding was in agreement with the presence of a promoter at the 5′ end of the *Mo*EF-Tu gene (Figure 8B), identified on the basis of the similarity with the corresponding region of other EF-Tu genes (*48*). A Pribnow box found within the coding region of the *Mo*EF-G gene was reported for *tufA* (*49*), whereas the −35 consensus (TTGCAG) was similar to that found in EF-Tu from *Bacillus stearothermophilus* and *Bacillus subtilis* (*19*). The 5′ untranslated region of the *Mo*EF-Tu gene is similar in length to the corresponding region of *E. coli* (*49*), *T. thermophilus* (*50*), and *T. aquaticus* (*48*) EF-Tu genes. Also in the case of *Mo*EF-Tu, a region encoding a possible hairpin loop is present at the 5′ flanking region, which might be important for translation efficiency (*50*). The transcription termination sequence is constituted by 12 bp inverted repeats, which allow the formation in the mRNA of a stem−loop structure (*19*).

The translated amino acid sequence of *Mo*EF-Tu contained the three sequence motifs typical of the GTP binding proteins (*51*). These were localized in the segments G¹⁹HVDHGK, D⁸¹CPG, and N¹³⁶KCD. *Mo*EF-Tu shares a sequence identity higher than 40% with other EF-Tus. A dendrogram, derived from the multiple alignment of the amino acid sequence of several EF-Tu/EF-1α, showed that psychrophilic *Moraxella* is more closely related to eubacteria rather than to eucarya or archaea; in addition, among eubacteria, *Moraxella* was closer to mesophiles rather than thermophiles or plant chloroplasts. Despite the high sequence identity among the eubacterial EF-Tus, codon usage in the corresponding genes is quite different. In fact, synonymous codon usage between *Ec*EF-Tu (*49*) and *Ta*EF-Tu (*48*) is similar, whereas it is different from *Mo*EF-Tu. On the contrary, it is noteworthy that *Mo*EF-Tu shows a codon usage similar to that found in *Ss*EF-1α (*23*).

A more appropriate evaluation of the biochemical properties and stability of *Mo*EF-Tu can be done only after its 3D structure will be solved.

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