Archaebacterial elongation factor Tu insensitive to pulvomycin and kirromycin

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A spermine-dependent, polyphenylalanine-synthesizing cell-free system having an optimum activity at 75–85°C, has been developed from the extremely thermoacidophilic archaebacterium *Caldariella acidophila*. The *C. acidophila* system is totally insensitive to the EF-Tu targeted antibiotics pulvomycin (at 40°C) and kirromycin (at 47–72°C) contrary to control systems derived from both mesophilic (*Escherichia coli*) and thermoacidophilic (*Bacillus acidocaldarius*) eubacteria. The archaebacterial EF-Tu-equivalent factor is also immunologically unrelated to eubacterial EF-Tu and does not cross react with antibodies against *Escherichia coli* EF-Tu. The pulvomycin and kirromycin reactions thus provide new phyletic markers for archaebacterial ancestry.

Archaebacteria *Elongation factor Tu* *Pulvomycin* *Kirromycin*

1. INTRODUCTION

Extant life forms are assumed to stem from the early divergence of a putative progenote into 3 lines of descent [1]: the eubacterial (encompassing most contemporary prokaryotes), the archaebacterial (comprising methanogenic, halophilic and thermoacidophilic microorganisms), and a third lineage (the urkaryotic descent) destined to give rise to eukaryotes. While a deep phylogenetic split exists between the two bacterial domains [2], the eukaryotic cell is viewed as a phylogenetic chimaera comprising both eubacterial and archaebacterial vestiges [2]. Indeed, two eukaryotic ribosomal proteins [3] and eukaryotic peptide translocation factor EF 2 [4] exhibit structural similarities with their archaebacterial counterparts.

Here, the specificity of the archaebacterial elongation factor equivalent to factors EF 1 (eukaryotic) and EF-Tu (eubacterial) has been probed by using the prokaryote-specific antibiotics pulvomycin [5] and kirromycin [6] which block EF-Tu activity prior to [5], or concomitant with [6], the ribosome requiring steps in protein synthesis. The extremely thermoacidophilic archaebacterium *Caldariella acidophila* (optimum growth temp. 87°C, pH 1–3) [7] has been investigated and compared to *Escherichia coli* and *Bacillus acidocaldarius* (a thermoacidophilic eubacterium growing optimally at 67°C and pH 1–3) [8]. The results show that the archaebacterial factor functionally homologous to EF-Tu cannot be assigned to the EF-Tu type and does not share antigenic determinants with EF-Tu.

Abbreviations: DTT, dithiothreitol; aa-tRNA, aminoacyl-tRNA; EF, elongation factor

2. MATERIALS AND METHODS

Crude ribosomes and 105 000 × *g* supernatants (S-100) from *C. acidophila*, strain MT 4, *B. acidocaldarius* and *E. coli*, RNAse I^-_{10} strain, were obtained as in [9]. The ribosomes were dissolved in a high-salt buffer (20 mM Tris–HCl (pH 7.3), 10 mM $MgO(Ac)_2$, 500 mM NH_4Cl, 0.5 mM DTT) and purified further by centrifugation in 12 ml Spinco tubes for 12 h at 105 000 × *g* over a 7 ml pad of 0.5 M sucrose in high-salt buffer. Soluble S-100 supernatant proteins were precipitated with 70% saturated $(NH_4)_2SO_4$, and were dissolved and

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dialysed in 20 mM Tris–HCl (pH 7.3), 1.0 mM $MgO(AC)_2$, 0.5 mM DTT, 10% glycerol. Mammalian 'run-off' ribosomes and 'pH 5 enzymes' were prepared from the liver of male Albino rats (Wistar strain) as in [10]; tRNA from all sources was isolated as in [11].

Poly(U)-directed *C. acidophila* cell-free systems (125 μl) contained (per ml): 1.0 mg ribosomes, 2.0 mg S-100 protein, 80 μg tRNA, 160 μg poly(U), 2.4 μmol ATP, 1.6 μmol GTP, 3.0 μmol spermine, 25 μmol Tris–HCl (pH 7.3), 19 μmol $MgO(Ac)_2$, 10 μmol NH_4Cl, 20 μmol DTT, 1.2 μCi of [U-^{14}C]-phenylalanine (spec.act. 513 mCi/mmol). The compositions of the poly(U)-directed *E. coli* and *B. acidocaldarius* assay mixtures were as for *C. acidophila* with the following modifications: spermine was omitted; NH_4Cl was either 70 mM (*E. coli*), or 20 mM (*B. acidocaldarius*); both systems contained creatinephosphate (10 mM) and creatinephosphate kinase (300 μg/ml); S-100 protein was present at 100–250 μg/ml. The composition of the rat liver poly(U)-directed cell-free system was as detailed in [10]. Hot trichloroacetic acid-insoluble radioactivity was assayed as in [12].

Escherichia coli EF-T and crystalline EF-Tu • GDP were purified as in [16]; *E. coli* EF-Ts was obtained from EF-T as in [13].

3. RESULTS AND DISCUSSION

The antibiotic sensitivity of the archaebacterial factor involved in aa-tRNA binding to ribosomes has been tested by using the poly(U)-directed *C. acidophila* cell-free system described in section 2. The principal features of the thermophile system, illustrated kinetically in fig.1, were as follows:

(i) At relatively high [Mg^{2+}] (18–20 mM) the synthetic activity was obligatorily dependent upon spermine (2–4 mM) at both high (75°C) and low (40°C) incubation temperatures.

(ii) The spermine activation of polyphenylalanine synthesis is drastically counteracted by NH_4^+ ions in excess of 10 mM, or by Tris–HCl buffer concentrations exceeding 25 mM;

(iii) The rates of peptide-bond formation display a stringent Arrhenius dependence upon temperature, a stable plateau level of maximum activity being only attained over the 75–85°C temperature interval.

Routinely the effects of pulvomycin and kirromycin on polyphenylalanine synthesis were assayed by using the least amount of S-100 protein required for optimum activity, and at either 40°C (pulvomycin) or 47°C (kirromycin) to rule out thermal degradation of the antibiotics. At 47°C the reference, *E. coli*, cell-free system was as active as at 37°C (not shown), while the *C. acidophila* system retained substantial activity (fig.1). The two antibiotics affect aa-tRNA binding to ribosomes by different mechanisms; pulvomycin impairs the ability of EF-Tu • GTP to form the ternary aa-

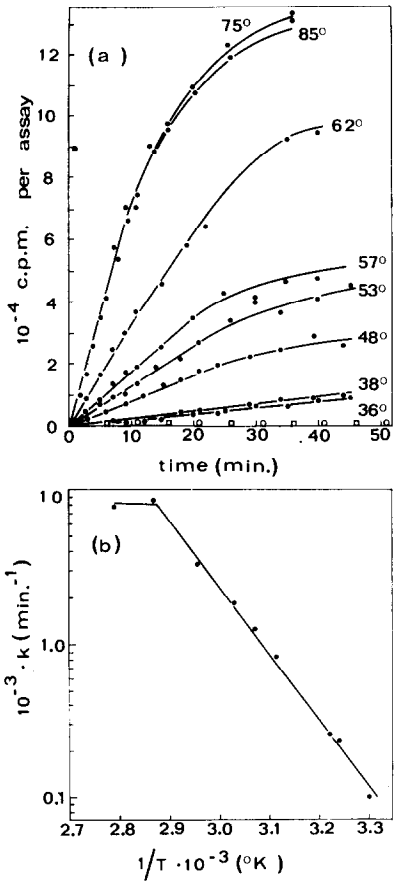

Fig.1. (a) Kinetics of polyphenylalanine synthesis by *C. acidophila* ribosomes: (•) complete assay system; (○) system lacking spermine incubated at either 75°C, or 40°C; (□) system containing 70 mM NH_4Cl incubated at 75°C. (b) Arrhenius plot of incorporation velocity *vs* incubation temperature. The rate constants (k) for polyphenylalanine synthesis were derived from the initial slopes of the kinetic curves in (a); k was calculated as hot trichloroacetic acid-insoluble radioactivity incorporated . min^{-1} . assay^{-1} (2.0 A_{260} units ribosomes).

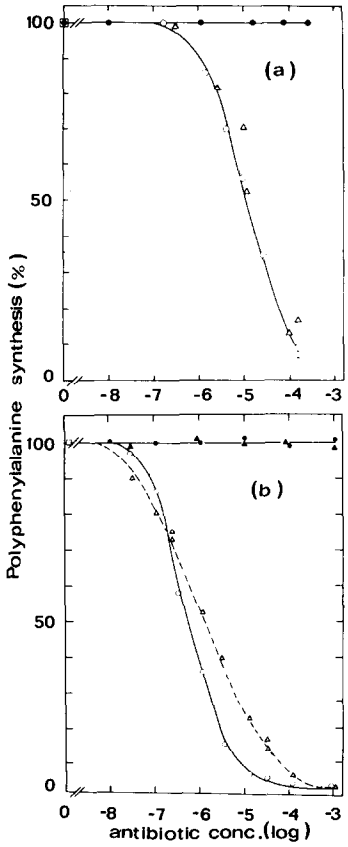

Fig.2. Effects of pulvomycin (a) and kirromycin (b) on the polyphenylalanine synthesizing capacities of: *E. coli* (○), *B. acidocaldarius* (△), *C. acidophila* (●) and rat liver (▲) cell-free systems. All reactions contained 2% ethanol (final conc.). The control assay systems had the following incorporating activities (radioactivity/mg ribosome after 45 min incubation): *E. coli* 80 × 10^4 cpm; *B. acidocaldarius*, 52–62 × 10^4 cpm; *C. acidophila*, 12–20 × 10^4 cpm; rat liver, 60 × 10^4 cpm. The incubation temperatures were 40°C (a) and 47°C (b).

tRNA • EF-Tu • GTP complex [5] whereas kirromycin binds stoichiometrically to EF-Tu and prevents its release from the ribosomal A site [6].

The polyphenylalanine synthesizing capacities of the reference *E. coli* and *B. acidocaldarius* systems are inhibited 95% and 87%, respectively, by pulvomycin (fig.2a) at 2.4 × 10^{-4} M (the upper solubility limit of the antibiotic in the presence of 2% ethanol), whereas the *C. acidophila* system is essentially unaffected; kirromycin (fig.2b) results

Table 1

Effect of kirromycin preheated for various time periods at 72°C on the polyphenylalanine synthesizing capacity of *E. coli* cell-free system

Kirromycin (10^{-4} M)	Kirromycin preincubation at 72°C (min)	Hot trichloroacetic acid-insoluble radioactivity (cpm/assay)
Absent		58 000
Present	0	880
Present	4	1000
Present	8	840
Present	30	862

Conditions for polyphenylalanine synthesis were as in section 2. Radioactivity refers to cpm in a 125 μl reaction mixture (2.0 A_{260} units ribosomes). Kirromycin solutions (10 mM) were heated in 98% (v/v) ethanol.

in complete inhibition of the two eubacterial systems at 2 × 10^{-5} M, while *C. acidophila* and rat liver cell-free systems are left unimpaired by antibiotic concentrations (10^{-3} M) which completely inhibit EF-Tu activity in poly(U)-programmed systems derived from kirromycin-resistant *E. coli* mutant strains [14].

Pulvomycin, also termed labilomycin [5], is easily degraded at high temperature and its effect on the *C. acidophila* system was only assayed at 40°C; kirromycin (10^{-4} M) was also tested at 72°C and found to be as ineffective as at 47°C (not shown). The lack of inhibition of the archaebacterial system by kirromycin at 72°C does not reflect antibiotic degradation since kirromycin samples preheated to 72°C for up to 30 min are fully active when assayed in the *E. coli* cell-free system at 37°C (table 1).

Further evidence that the antibiotic-resistant archaebacterial elongation factor and eubacterial EF-Tu are structurally distinct entities is provided by the experiment in fig.3 in which antibodies raised against *E. coli* EF-T (the 1:1 complex of EF-Tu and EF-Ts) were assayed against S-100 proteins of *E. coli*, *B. acidocaldarius* and *C. acidophila* strains MT 4 and MT 3 (the latter is a less thermophilic strain growing optimally at 75°C); the precipitin arcs corresponding to the individual EF-Tu and EF-Ts components of the EF-T complex were identified by using *E. coli* crystalline EF-Tu • GDP

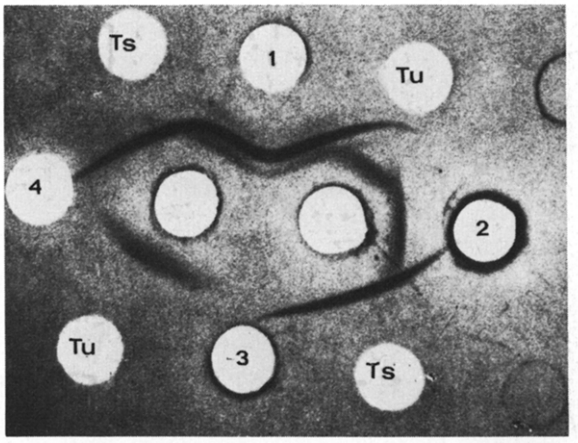

Fig.3. Double-diffusion test of soluble proteins of *E. coli*, *B. acidocaldarius* and *C. acidophila*, with antibodies against *E. coli* EF-T complex. The centre wells contained immune serum against *E. coli* EF-T (1.4 mg IgG protein). The peripheral wells contained 20 μg *E. coli* EF-Tu • GDP (Tu); 6.0 μg *E. coli* EF-Ts (Ts); 0.75 mg *E. coli* S-100 protein (1); 1.6 mg *B. acidocaldarius* S-100 protein (2); 2.6 mg *C. acidophila* S-100 protein (MT-4 strain) (3); 2.6 mg *C. acidophila* S-100 protein (MT-3 strain) (4). *E. coli* EF-T specific immune sera were raised in rabbits; EF-T (0.8 mg) was given subcutaneously in complete Freund's adjuvant. Boosters, (0.5 mg EF-T), were repeated 6 times over 4 months. The rabbits were bled 4 days after the last booster; IgG were precipitated with 40% saturated $(NH_4)_2SO_4$ and further purified by 2 cycles of precipitation with 33.3% saturated $(NH_4)_2SO_4$. The IgG were lastly dissolved in 20 mM phosphate buffer, (pH 7.3), 150 mM NaCl, 45% glycerol and dialysed against the same solvent.

and electrophoretically homogeneous EF-Ts as the reference antigens. The Ouchterlony double reactions in fig.3 show that common antigenic determinants are shared by the EF-Tu factors of such distantly related eubacteria as *E. coli* and *B. acidocaldarius*; by contrast, no immunological homology appears to exist between eubacterial EF-Tu and the functionally homologous factor of either the MT 4 or the MT 3 strains of *C. acidophila*. Parenthetically, the immune test also shows that in the eubacterial line of descent EF-Ts has been less stringently conserved than EF-Tu, since no antigenic determinants combining with *E. coli* anti-EF-Ts antibodies are discernible among the soluble proteins of *B. acidocaldarius*.

The combined evidence in fig.2 and 3 allows the conclusion that the archaebacterial factor involved in the binding of aa-tRNA to ribosomes cannot be assigned to the EF-Tu type. The possibility that the observed heterologies reflect structural adaptive changes of an otherwise EF-Tu type factor to an unusually hot niche (rather than ancestry of the thermoacidophilic archaebacterial strain) can be discounted:

(1) EF-Tu from the extremely thermophilic eubacterium *Thermus thermophilus*, strain HB 8 (optimum growth temp. 85°C) [16] is as sensitive to kirromycin as *E. coli* EF-Tu (Y. Kaziro, personal communication).

(2) Evidence has been cited [17] that a high-salt-dependent, EF-Tu-equivalent factor from the mesophilic archaebacterium *Halobacterium cutirubrum* is also resistant to kirromycin.

Therefore, we feel that the pulvomycin and kirromycin reactions provide new phyletic markers for archaebacterial ancestry. Together with previous data on the ADP-ribosylability of the archaebacterial EF G-equivalent factor by *Diphtheria* toxin [4], these results support the notion that in the thermoacidophilic branch of archaebacteria both the aa-tRNA binding to ribosomes and the peptide translocation step are assisted by lineage-specific components. Of the 2 components involved, the peptide translocase is structurally related to that of the eukaryotic cytosol, at least insofar as it provides the prerequisite amino acid sequence around the modified hystidine residue which is ADP-ribosylated by the *Diphtheria* toxin reaction [18]. By contrast, the relatedness of archaebacterial EF-Tu equivalent factor to eukaryotic EF-1 remains undecided; the evidence that both above factors lack affinity for kirromycin and pulvomycin does not imply per se that they are structurally related. To assess this point the archaebacterial EF-Tu equivalent factor and eukaryotic EF-1 are presently being compared by peptide mapping and immuno-crossreactivity tests.

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