JOURNAL OF BACTERIOLOGY, Mar. 1996, p. 1762–1765 Vol. 178, No. 6
0021-9193/96/$04.00+0

NOTES

Sensitivity of Ribosomes of the Hyperthermophilic Bacterium *Aquifex pyrophilus* to Aminoglycoside Antibiotics

MAURIZIO BOCCHETTA,[1]† R. HUBER,[2] AND PIERO CAMMARANO[1]*

Istituto Pasteur Fondazione Cenci-Bolognetti, Dipartimento Biopatologia Umana, Sezione di Biologia Cellulare, Universita' di Roma "La Sapienza," Policlinico Umberto I, 00161 Rome, Italy,[1] and Lehrstuhl für Mikrobiologie, Universität Regensburg, D8400-Regensburg, Federal Republic of Germany[2]

Received 21 July 1995/Accepted 4 January 1996

A poly(U)-programmed cell-free system from the hyperthermophilic bacterium *Aquifex pyrophilus* has been developed, and the susceptibility of *Aquifex* ribosomes to the miscoding-inducing and inhibitory actions of all known classes of aminoglycoside antibiotics has been assayed at temperatures (75 to 80°C) close to the physiological optimum for cell growth. Unlike *Thermotoga maritima* ribosomes, which are systematically refractory to all known classes of aminoglycoside compounds (P. Londei, S. Altamura, R. Huber, K. O. Stetter, and P. Cammarano, J. Bacteriol. 170:4353–4360, 1988), *Aquifex* ribosomes are susceptible to all of the aminoglycosides tested (disubstituted 2-deoxystreptamines, monosubstituted 2-deoxystreptamines, and streptidine compounds). The significance of this result in light of the *Aquifex* and *Thermotoga* placements in phylogenetic trees of molecular sequences is discussed.

Aquifex pyrophilus is a microaerophilic, gram-negative, hydrogen-oxidizing, hyperthermophilic bacterium thriving optimally at 85°C (11). It differs from other bacteria in possessing unique glycerol-diether membrane core lipids (11) and ribosomes having an unusually large protein complement (1). Phylogenetic trees of 16S rRNA (5) and protein synthesis elongation factor G (EF-G) (3) sequences indicate that *A. pyrophilus* has a particularly deep lineage, branching somewhat more deeply than *Thermotoga maritima.*

We have reported elsewhere (14) that *T. maritima* (optimum growth temperature, 85°C) differs from other bacteria in being systematically insensitive to all known groups of aminoglycoside antibiotics, both in vivo and in vitro, even though *Thermotoga* ribosomes do possess all of the 16S rRNA sequence elements that are required for antibiotic action. Given the apparent vicinity of the divergences of *A. pyrophilus* and *T. maritima* in the 16S rRNA and EF-G trees, we wanted to know whether the unprecedented antibiotic sensitivity spectrum of *Thermotoga* ribosomes extends to neighboring deeply branching bacterial phyla or is a unique anomaly of the order *Thermotogales*. To investigate this point, we have developed a poly(U)-programmed cell-free system from *A. pyrophilus*. The results of in vitro assays show that *A. pyrophilus* differs from *T. maritima*, and resembles the classically recognized bacteria, in being systematically susceptible to all of the aminoglycosides tested.

Development of an *A. pyrophilus* poly(Phe)-synthesizing system. *Aquifex* cells (Deutsche Sammlung Mikroorganismen) (DSM 6858) were grown as described previously (11) and disrupted by using a French pressure cell in a medium (extraction buffer) containing 20 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, and 50 mM NH_4Cl. After addition of DNase (2.5 μg/ml) and removal of cell debris, ribosomes and a ribosome-free supernatant were obtained by centrifugation of the lysate in a Spinco Beckman Ti50 rotor operated at 40,000 rpm for 3 h. The crude ribosomes were dissolved in extraction buffer containing 500 mM NH_4Cl (high-salt buffer) and purified by centrifugation through a 7.0-ml pad of 0.5 M sucrose made up in the high-salt buffer (13, 14). Soluble proteins were precipitated from the ribosome-free supernatant with 70% saturated $(NH_4)_2SO_4$ and dialyzed in 20 mM Tris-HCl (pH 7.4)–1.0 mM magnesium acetate–0.5 mM β-mercaptoethanol. The *A. pyrophilus* system for poly(Phe) synthesis was developed gradually, but the final reaction mixture contained (per milliliter) 50 μmol of Tris-HCl (pH 7.4), 10 μmol of NH_4Cl, 10 μmol of magnesium acetate, 2.0 μmol of spermine, 2.0 μmol of ATP, 1.0 μmol of GTP, 5.0 μmol of β-mercaptoethanol, 140 μg of poly(U), 143 pmol (6.0 A_{260} units) of ribosomes, 120 μg of the ribosome-free supernatant protein, 250 μg of *Sulfolobus solfataricus* tRNA (the same preparation used previously to optimize the *Thermotoga* cell-free system [14]), and 4.0 μCi of L-[^{14}C]phenylalanine (522 mCi/mmol). For assays of misreading the poly(U)-directed system was as described above except that it contained 2.0 nmol of L-phenylalanine and 4.0 μCi of L-[^{14}C]leucine (344 mCi/mmol).

Synthetic capacity of the *Aquifex* cell-free system. The dependency of poly(Phe) synthesis upon temperature is illustrated kinetically in Fig. 1. Under optimized ionic conditions, an average synthetic capacity of four or five phenylalanine residues per ribosome (in 40 min) was attained at 70 to 80°C, which is close to the physiologically optimal temperature for cell growth (Fig. 1A). As Fig. 1B shows, however, only about 30% of the 50S and 30S subunit particles participated in poly(Phe) synthesis, thus giving an average synthetic capacity of 12 to 15 residues per active ribosome.

Titration curves of spermine, Mg^{2+}, and monovalent cations for optimal poly(Phe) synthesis are illustrated in Fig. 2; with

* Corresponding author. Mailing address: Dipt. Biopatologia Umana, Sezione Biologia Cellulare, Policlinico Umberto I, Viale Regina Elena 324, 00161, Roma, Italy. Phone: 39-6-4940609. Fax: 39-6-4462891. Electronic mail address: piero@dbu.uniromal.it.

† Present address: The University of Illinois at Chicago, Dept. of Medicinal Chemistry, College of Pharmacy, Chicago, IL 60612.

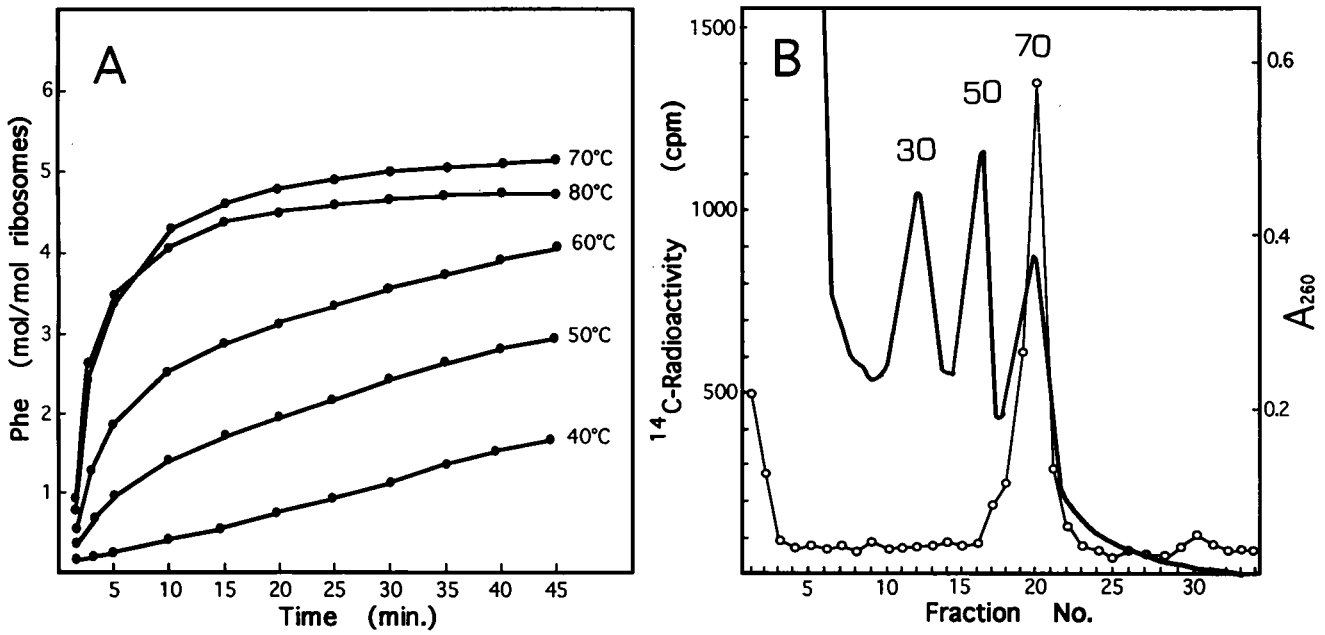

FIG. 1. (A) Temperature dependence of poly(Phe) synthesis. Hot trichloroacetic acid-insoluble radioactivity was determined as described previously (13). (B) Sucrose density gradient distribution of [^{14}C]poly(Phe) chains on *A. pyrophilus* ribosomes after a 40-min incubation of the cell-free system at 80°C. The reaction mixture (200 μl; 1.2 A_{260} units of ribosomes) contained 50 mM triethanolamine-HCl (pH 7.4) instead of Tris-HCl and contained 10 mM KCl instead of NH_4Cl (13). After fixation with formaldehyde to prevent pressure-induced dissociation of 70S monomers during centrifugation (13), the sample was layered on an 11.5-ml 10 to 30% (wt/vol) sucrose density gradient made in 50 mM triethanolamine-HCl (pH 7.4)–10 mM magnesium acetate. The gradient was centrifuged in a Spinco Beckman SW41 rotor operated at 35,000 rpm and 4°C for 3 h. Fractions (0.3 ml each) were collected to measure hot trichloroacetic acid-insoluble radioactivity (open circles) while continuously monitoring A_{254}) (solid line); ^{14}C radioactivity was measured with a 90% counting efficiency by using a Beckman LS 1801 radiation spectrometer. Sedimentation values (S) are indicated above the absorbance peaks.

poly(U) as the messenger, *Aquifex* ribosomes were obligatorily dependent upon a high spermine concentration (2.0 mM) and did not tolerate NH_4^+ concentrations higher than 20 mM. The comparative data in Table 1 show that the polyamine and ionic requirements of the *Aquifex* system differ markedly from those of *T. maritima* (0.5 mM spermine and 100 mM NH_4Cl) and resemble those of systems prepared from members of the *Crenarchaeota* (*S. solfataricus*, *Desulfurococcus mobilis*, and *Thermoproteus tenax*) (13). The role played by spermine is unclear, however, as spermine is not needed for cell-free translation of natural mRNAs by *Sulfolobus* ribosomes (16). For a rigorous comparison with the *Thermotoga* system, *S. solfataricus* tRNA was used instead of *Aquifex* tRNA. In fact, the archaeal (*Sulfolobus*) tRNA was routinely used to test the antibiotic sensitivity of *Thermotoga* ribosomes and was shown to effectively replace the homologous (*Thermotoga*) tRNA (14).

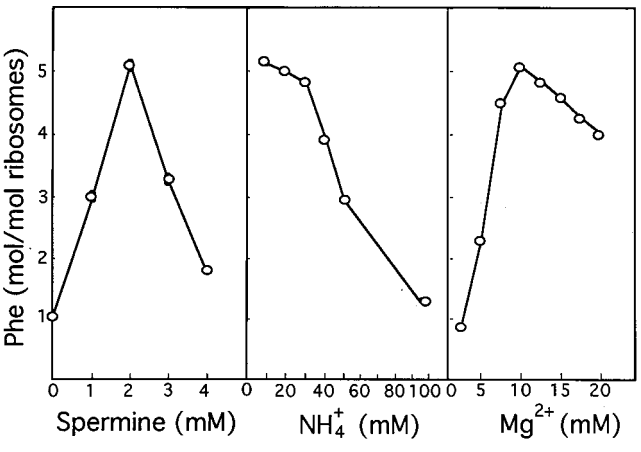

FIG. 2. Ionic and polyamine requirements of *A. pyrophilus* ribosomes at 75°C. (Left) Spermine dependence of poly(Phe) synthesis in the presence of 10 mM NH_4Cl and 10 mM Mg^{2+}; (middle) NH_4^+ dependence of poly(Phe) synthesis in the presence of 10 mM Mg^{2+} and 2.0 mM spermine; (right) Mg^{2+} dependence of poly(Phe) synthesis in the presence of 2.0 mM spermine and 10 mM NH_4Cl.

Antibiotic sensitivity assays. The following compounds were tested for their abilities to inhibit poly(Phe) synthesis (Fig. 3A to C) and to promote translational misreading (Fig. 3D to F): neomycin and paromomycin (4,5-disubstituted 2-deoxystreptamines), kanamycin (a 4,6-disubstituted 2-de-

TABLE 1. Ionic and polyamine requirements of poly(U)-directed cell-free systems from hyperthermophilic euryarchaeotes (*Thermococcus celer* and *Pyrococcus woesei*), chrenarchaeotes (*D. mobilis*, *T. tenax*, and *S. solfataricus*), and bacteria[a]

Organism	Requirement (mM) for:			Activity (Phe/ribosome)[b]	Reference
	NH_4^+	Mg^{2+}	Spermine		
T. celer	100	15	0.0	8–10	13
P. woesei	100	10	0.5	10–15	7
D. mobilis	10	18	3.0	10–15	13
T. tenax	10	18	3.0	3–4	13
S. solfataricus	10	18	3.0	30–40	13
A. pyrophilus	10	10	2.0	5 (12)	This report
T. maritima	100	10	0.5	7 (15)	3

[a] For all organisms, the temperature optimum was 75 to 80°C.

[b] Poly(Phe) synthetic capacities are given on a total-ribosome basis; numbers in parentheses indicate the capacities calculated on the basis of the active-ribosome fractions.

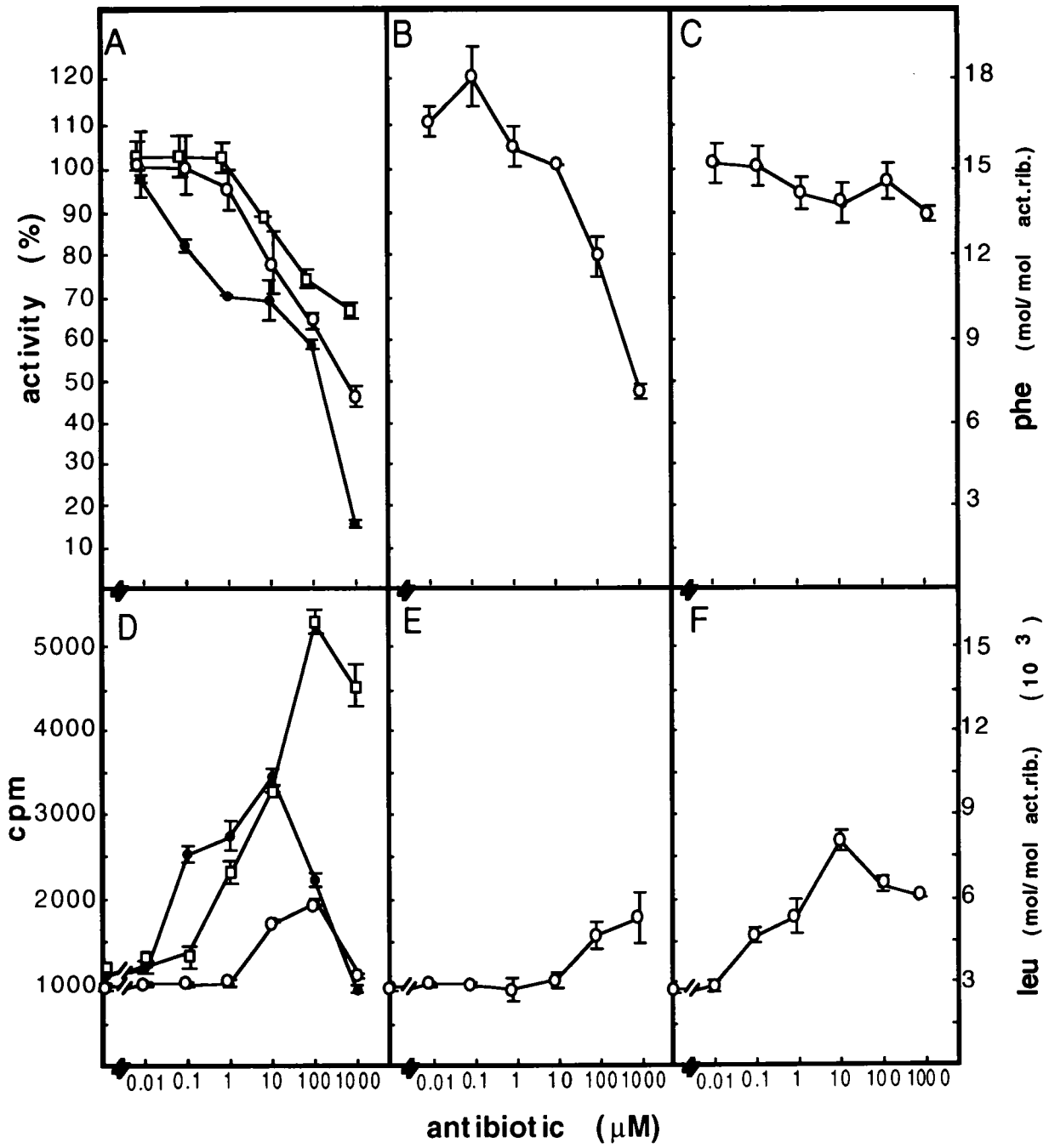

FIG. 3. (A and D) Effects on poly(Phe) synthesis (A) and [^{14}C]leucine incorporation (D) of the disubstituted 2-deoxystreptamine compounds kanamycin (squares), paromomycin (open circles), and neomycin (filled circles). (B and E) Effects on poly(Phe) synthesis (B) and [^{14}C]leucine incorporation (E) of the monosubstituted 2-deoxystreptamine hygromycin. (C and F) Effects on poly(Phe) synthesis (C) and [^{14}C]leucine incorporation (F) of the streptidine compound streptomycin. Bars indicate standard deviations based on the results of five replicate assays. The antibiotics used were the same stock solutions used previously (14) for testing the aminoglycoside sensitivity of *Thermotoga* ribosomes; kanamycin was a 95:5 (wt/wt) mixture of kanamycins A and B, and neomycin was a mixture of the B (92%) and A (8%) components. The right ordinate shows amino acid incorporations per active ribosome (act.rib.) as determined in Fig. 1.

oxystreptamine), hygromycin B (a monosubstituted 2-deoxystreptamine), and streptomycin (a member of the streptidine group). Since all of these drugs retain full activity upon prolonged incubation at temperatures of up to 80°C (6), the antibiotic sensitivity of *Aquifex* ribosomes could be assayed at the optimum temperature for in vitro translation. The results in Fig. 3A to C show that all of the compounds tested, except streptomycin, inhibit poly(Phe) synthesis, although to various degrees (from 30 to 90% at 10^{-3} M); streptomycin was ineffective even at a concentration (10^{-3} M [approximately 600 μg/ml]) known to result in a 60% inhibition of poly(Phe) synthesis on *Bacillus stearothermophilus* ribosomes (14).

The effects of the aminoglycosides on the accuracy of poly(U) translation were assayed by measuring changes in the

incorporation of a noncognate amino acid ([^{14}C]leucine) (Fig. 3D to F). All of the compounds tested increased the error frequency in tRNA selection, although to different degrees (from twofold for paromomycin to fivefold for kanamycin). The decrease of [^{14}C]leucine incorporation seen at high antibiotic concentrations most likely reflects inhibition of all protein synthesis, whether normal or ambiguous, as a result of drug binding to low-affinity sites (10). In two cases (hygromycin and streptomycin), induction of miscoding and inhibition of poly(Phe) synthesis were dissociated: streptomycin promoted an appreciable increase of [^{14}C]leucine incorporation while leaving poly(Phe) synthesis unaffected; conversely, hygromycin inhibited poly(Phe) synthesis by about 50% (at 10^{-3} M) but had practically no effect on incorporation of [^{14}C]leucine.

The significance of the opposite antibiotic sensitivity spectra exhibited by the *A. pyrophilus* (this report) and *T. maritima* (14) ribosomes depends on the relative phylogenetic depths of the two hyperthermophilic lineages. If the evolutions of the 16S rRNA (5) and the EF-G (3) marker molecules provide a trustworthy picture of organismal relationships, then *A. pyrophilus* and *T. maritima* constitute the deepest and the second-deepest bacterial radiations, respectively. In this phylogenetic perspective, the present results argue against the possibility that the behavior of *Thermotoga* ribosomes is ancestral, with aminoglycoside sensitivity being a later development in the evolution of bacterial ribosomes; the lack of sensitivity of *Thermotoga* ribosomes to all classes of aminoglycoside compounds (14) could be interpreted only as indicating unique changes of antibiotic-binding sites incurred by the 30S subunit particles during the separate evolutionary course of the order *Thermotogales*. Although unprecedented among bacteria, this is a common event among the archaea, which display erratic antibiotic sensitivity spectra reflecting a nonsystematic instability of antibiotic-binding sites (reviewed in reference 2).

However, two reservations should be borne in mind. First, the rooting of the bacterial 16S rRNA and EF-G trees at the *Aquifex-Thermotoga* level and the branching of *A. pyrophilus* more deeply than *T. maritima* (rather than the reverse), although consistently inferred by alternative tree-making methods, cannot be firmly asserted on statistical (bootstrap confirmation) grounds (3, 5). Second, alternative placements of the two hyperthermophilic lineages are predicted by evolutionary trees of RNA polymerase subunit and glutamine synthetase I sequences. The RNA polymerase-derived phylogeny (12) has *T. maritima* as the most deeply rooted free-living bacterium, with *A. pyrophilus* being a later divergence situated between the gram-positive bacteria with low G+C contents (e.g., *Mycobacterium leprae*) and the members of the class *Proteobacteria*; this topology would indeed support the possibility that the antibiotic sensitivity spectrum exhibited by *Thermotoga* ribosomes is ancestral. On the other hand, phylogenies based on glutamine synthetase I (4, 17) reveal unexpected deviations from the 16S rRNA paradigm that could be most parsimoniously interpreted (4) by taking *T. maritima* to be a member of the gram-positive bacteria with low G+C in sharp contrast to the *Thermotoga* placement inferred from evolutionary trees of EF-Tu (8, 15), EF-G (9, 18), and 16S rRNA (5, 19, 20) sequences. Accordingly, we feel that a more informed interpretation of the different antibiotic sensitivity spectra exhibited by the two hyperthermophilic bacteria must await the analysis of a larger set of molecular sequence data.

This work was supported by MURST and by the CNR target project on BTBS.

REFERENCES

1. **Acca, M., M. Bocchetta, E. Ceccarelli, R. Creti, K. O. Stetter, and P. Cammarano.** 1994. Updating mass and composition of archaeal and bacterial ribosomes. Archaeal-like features of ribosomes from the deep branching bacterium *Aquifex pyrophilus*. Syst. Appl. Microbiol. **16:**629–637.
2. **Amils, R., P. Cammarano, and P. Londei.** 1993. Translation in archaea, p. 393–438. *In* M. Kates, D. J. Kushner, and A. T. Matheson (ed.), Biochemistry of archaebacteria (archaea). Elsevier, New York.
3. **Bocchetta, M., E. Ceccarelli, R. Creti, A. M. Sanangelantoni, O. Tiboni, and P. Cammarano.** Arrangement and nucleotide sequence of the gene (*fus*) encoding elongation factor G (EF-G) from the hyperthermophilic bacterium *Aquifex pyrophilus*: phylogenetic depth of hyperthermophilic bacteria inferred from analysis of EF-G/*fus* sequences. J. Mol. Evol., in press.
4. **Brown, J. R., Y. Masuchi, F. T. Robb, and W. F. Doolittle.** 1994. Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. J. Mol. Evol. **38:**566–576.
5. **Burggraf, S., G. J. Olsen, K. O. Stetter, and C. R. Woese.** 1992. A phylogenetic analysis of *Aquifex pyrophilus*. Syst. Appl. Microbiol. **15:**352–356.
6. **Cammarano, P., A. Teichner, P. Londei, M. Acca, B. Nicolaus, J. L. Sanz, and R. Amils.** 1985. Insensitivity of archaebacterial ribosomes to protein synthesis inhibitors. Evolutionary implications. EMBO J. **4:**811–816.
7. **Catani, V., S. Altamura, and P. Londei.** 1990. Antibiotic sensitivity of the ribosomes of the ultrathermophilic archaebacterium *Pyrococcus woesei*. FEMS Microbiol. Lett. **70:**285–290.
8. **Cousineau, B., C. Cerpa, J. Lefebvre, and R. Cedergren.** 1992. The sequence of the gene encoding elongation factor Tu from *Chlamydia trachomatis* compared with those of other organisms. Gene **120:**33–41.
9. **Creti, R., E. Ceccarelli, M. Bocchetta, A. M. Sanangelantoni, O. Tiboni, P. Palm, and P. Cammarano.** 1994. Evolution of translational elongation factor (EF) sequences: reliability of global phylogenies inferred from EF-1α (Tu) and EF-2(G) proteins. Proc. Natl. Acad. Sci. USA **91:**3255–3259.
10. **Davies, J., and B. D. Davies.** 1968. Misreading of amino acid codewords induced by aminoglycoside antibiotics. The effect of drug concentration. J. Biol. Chem. **243:**3312–3316.
11. **Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. Konig, R. Rachel, I. Rockinger, H. Fricke, and K. O. Stetter.** 1992. *Aquifex pyrophilus* gen nov., sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst. Appl. Microbiol. **15:**340–351.
12. **Klenk, H.-P., P. Palm, and W. Zillig.** 1994. DNA-dependent RNA polymerases as phylogenetic marker molecules, p. 138–147. *In* F. Pfeiffer, P. Palm, and K. H. Schleifer (ed.), Molecular biology of archaea. Fischer Verlag, New York.
13. **Londei, P., S. Altamura, P. Cammarano, and L. Petrucci.** 1986. Differential features of ribosomes and of poly(U)-programmed cell-free systems derived from sulphur-dependent archaebacterial species. Eur. J. Biochem. **157:**455–462.
14. **Londei, P., S. Altamura, R. Huber, K. O. Stetter, and P. Cammarano.** 1988. Ribosomes of the extremely thermophilic eubacterium *Thermotoga maritima* are uniquely insensitive to the miscoding-inducing action of aminoglycoside antibiotics. J. Bacteriol. **170:**4353–4360.
15. **Ludwig, W., M. Weizenegger, D. Betzl, E. Leidel, T. Lenz, A. Ludvigsen, D. Mollendorf, P. Wenzig, and K. H. Schleifer.** 1990. Complete nucleotide sequence of seven eubacterial genes coding for the elongation factor Tu: functional, structural and phylogenetic evaluations. Arch. Microbiol. **153:** 241–247.
16. **Ruggero, D., R. Creti, and P. Londei.** 1993. *In vitro* translation of archaeal natural mRNAs at high temperature. FEMS Microbiol. Lett. **107:**89–94.
17. **Tiboni, O., P. Cammarano, and A. M. Sanangelantoni.** 1993. Cloning and sequencing of the gene encoding glutamine synthetase I from the archaeum *Pyrococcus woesei*: anomalous phylogenies inferred from analysis of archaeal and bacterial glutamine synthetase I sequences. J. Bacteriol. **175:**2961–2969.
18. **Tiboni, O., R. Cantoni, R. Creti, P. Cammarano, and A. M. Sanangelantoni.** 1991. Phylogenetic depth of *Thermotoga maritima* inferred from analysis of the *fus* gene: amino acid sequence of elongation factor G and organization of the *Thermotoga str* operon. J. Mol. Evol. **33:**142–151.
19. **Van De Peer, Y., J. M. Neefs, P. De Rijk, P. De Vos, and R. De Wachter.** 1994. About the order of divergence of the major bacterial taxa during evolution. Syst. Appl. Microbiol. **17:**32–38.
20. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.