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Studies on rDNA from the extreme thermophilic eubacterium *Thermus thermophilus* HB8

The 23 S rDNA region D

Roland K. Hartmann, Detlef W. Vogel, Bernd Kröger, Norbert Ulbrich and Volker A. Erdmann

Freie Universität Berlin, Institut für Biochemie, Thielallee 63, 1000 Berlin 33, Germany

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23 S ribosomal ribonucleic acid gene from the extreme thermophile eubacterium *Thermus thermophilus* HB8 has been cloned in pBR322, and the nucleotide sequence of region D has been determined, which encompasses 873 nucleotides at the 3'-end of the RNA. We compare the primary and secondary structure of this region with the respective part of the 23 S rRNA from *Escherichia coli* and *Bacillus stearothermophilus*. A high level of structural conservation can be observed, throughout the RNA domain, albeit the usage of G/C basepairs is substantial even in comparison with another thermophilic eubacterium *B. stearothermophilus*. It is surprising that, in contrast to the usage of $^{3'}$U-G$^{5'}$, the occurrence of $^{3'}$G-U$^{5'}$ is comparable in *E. coli* as well as in *B. stearothermophilus* and *T. thermophilus*. Furthermore, it is most remarkable that the use of $^{3'}$A-U$^{5'}$ and $^{3'}$U-A$^{5'}$ is, compared to *E. coli*, only slightly reduced in *B. stearothermophilus*, but drastically decreased in *T. thermophilus*.

23 S rRNA; 23 S rRNA structure; rDNA; (*Thermus thermophilus* HB8)

1. INTRODUCTION

Elucidation of the structure of ribosomal ribonucleic acids is of fundamental importance to the understanding of both the assembly and function of the ribosomal particles and for studying the evolution of organisms. The application of the comparative sequence approach has been primarily responsible for the secondary structural models that have been published for the large ribosomal ribonucleic acids [1–3].

Here, we describe the primary and secondary structure of domain D of 23 S rRNA from the extreme thermophilic eubacterium *Thermus thermophilus* HB8, as derived from DNA sequencing data. As a part of this project we have reported previously on the physical organization of the rRNA genes and on the primary and secondary structures of the 3'-distal portion of the two rDNA operons [4–6].

Correspondence address: N. Ulbrich, Freie Universität Berlin, Institut für Biochemie, Thielallee 63, 1000 Berlin 33, Germany

2. MATERIALS AND METHODS

T. thermophilus HB8 (ATCC 27634) cells were grown at 70°C as in [7]. rDNA carrying fragments, 6.75 and 7.00 kb in size, which were identified by genomic hybridization, were cloned into the vectors pBR322 and pSP65 resulting in the recombinant plasmids pTT675 and pTT700 [4]. To determine the exact location of rDNA carrying portions within the recombinant plasmids, they were digested with the restriction endonucleases *Pst*I, *Bss*HII, *Aha*III, *Sac*II, *Pvu*II, *Hin*dIII, *Xba*I, *Kpn*I and *Bam*HI [4]. To analyse the primary structure of DNA, fragments were cloned into the double-stranded replicative form of the

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M13 bacteriophages mp8/9 or mp18/19. The isolation of the recombinant phages was essentially as described [8] with the following modifications: 2 ml of 2 × TY media were inoculated with 20 μl of an overnight culture of *E. coli* BMH 71/18 [9] and 20 μl of recombinant phage supernatant. After 5 h incubation at 37°C, the phage supernatants were collected by repeated low-speed centrifugation for 5 min at 15000 × *g*. To 1.5 ml phage supernatant, 150 μl of 40% polyethylene glycol 6000 (PEG, Merck) and 150 μl of 5 M NaCl were added. The mixture was incubated on ice for 30 min, followed by a low-speed centrifugation at 15000 × *g* for 5 min. Phage sediments were resuspended in 200 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), followed by the addition of 12 μl of 40% PEG and 36 μl of 5 M NaCl. Samples were incubated at room temperature for 30 min and centrifuged as described above. The phages resuspended in 200 μl TE buffer were extracted once with phenol, and finally once with phenol/chloroform (1:1). Phage DNA was collected by ethanol precipitation, washed with 70% ethanol and dissolved in 14 μl TE buffer; 2–4 μl of this DNA solution were used for the sequencing reactions, which were performed according to the dideoxynucleotide chain termination method [10]. The restriction endonucleases *Kpn*I, *Xba*I, *Bam*HI, *Hin*dIII, *Pvu*II, *Pst*I, were purchased from Boehringer, Mannheim, and *Bss*HII, *Sac*II and *Aha*III from Biolabs. The T_4 DNA ligase was purchased from Bethesda Research Laboratories.

3. RESULTS AND DISCUSSION

T. thermophilus HB8 was chosen for this study because it is a thermophile. Some understanding of the structural basis of thermal stability of its ribosomes may emerge from comparison with the rRNA of mesophiles, such as *E. coli* [11] and the thermophile Gram-positive eubacterium *B. stearothermophilus* [12]. This information could contribute to the prediction of the stability of RNA structures by thermodynamic criteria.

The recombinant rDNA plasmid pTT675 (fig.1) was used as a source of restriction fragments for DNA sequencing. A detailed physical map allowed us to isolate definite portions coding for the 3′-terminal part of the 23 S rDNA [4]. These portions were digested with several restriction endonucleases, resulting in fragments, which were cloned into M13 phages mp8/mp9 and mp18/mp19 respectively and analysed by enzymatic sequencing [10] of both strands. The gene coding for the 23 S rRNA could be identified by virtue of sufficient homology to 23 S rRNAs already described [11,12]. The complete nucleotide sequence of region D, aligned with that of *E. coli* and *B. stearothermophilus*, is shown in fig.2.

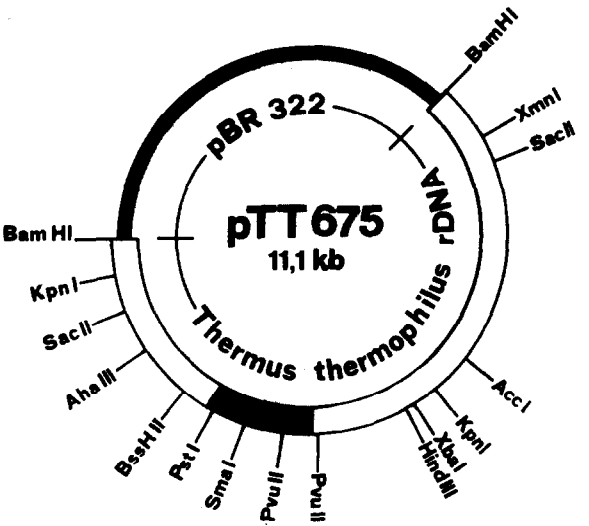

Fig.1. Schematic representation of the recombinant plasmid pTT675. The sequenced portion is marked as a black box within the cloned rDNA region. The plasmid contains various *Sma*I sites, but only the one important for subcloning is indicated.

One obvious expectation of an extreme thermophilic eubacterium is that it has a greater extent of G/C basepairing in its secondary structure. Indeed, the usage of G/C basepairs is substantial even in comparison with another thermophilic eubacterium *B. stearothermophilus* (table 1A). It is also surprising that in contrast to the usage of 3′U-

Fig.2. Nucleotide sequence of a 23 S rRNA region D from *T. thermophilus* HB8, inferred from its gene sequence. The sequence of the respective part of the 23 S rRNA from the rrnB transcriptional unit of *E. coli* [11] and from *B. stearothermophilus* [3] is shown in comparison. Hyphens are shown where nucleotides are absent in the sequence from one organism. Nucleotides not conserved in all three sequences are indicated by an asterisk.

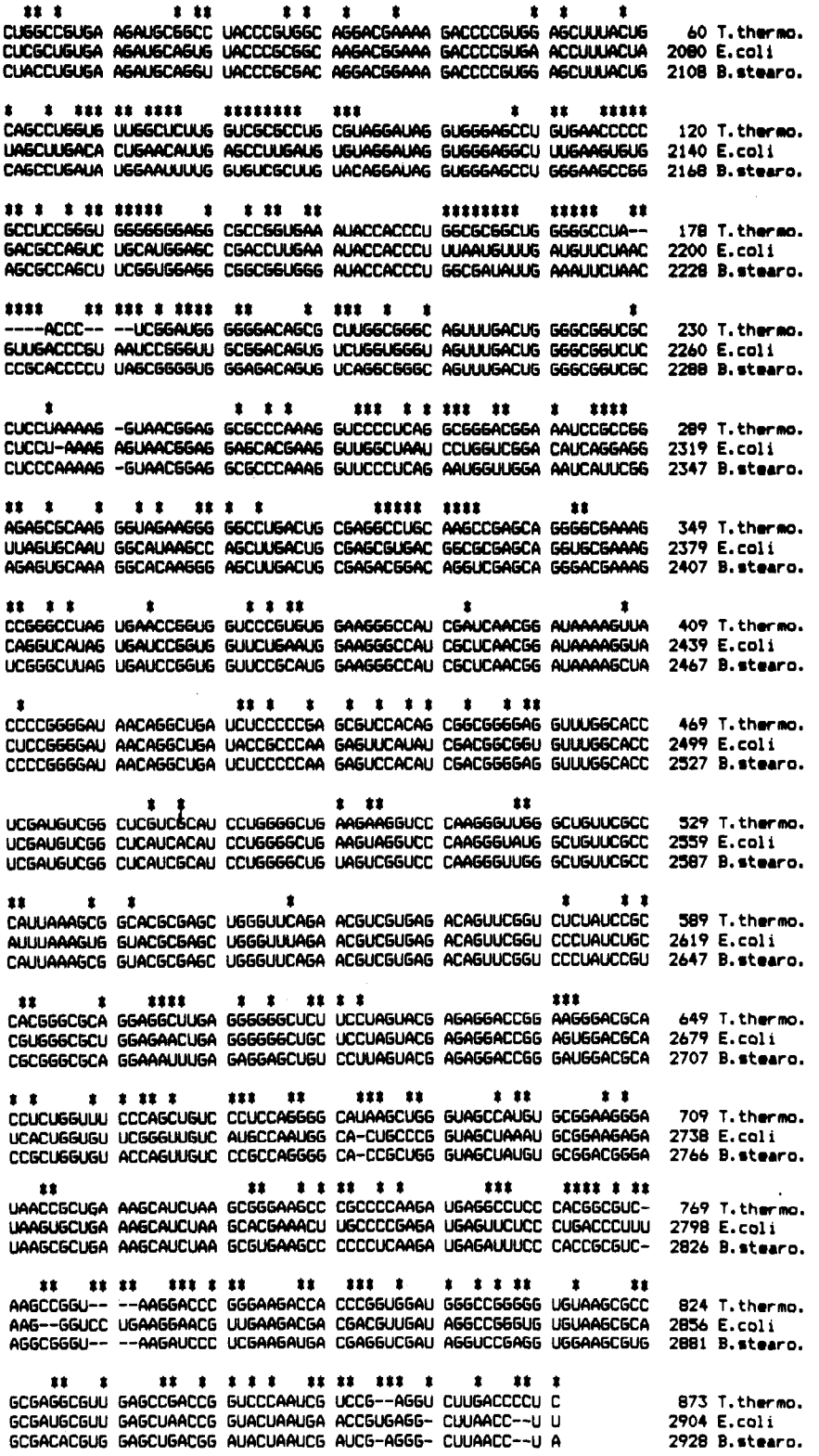
CUGGCCGUGA AGAUGCGGCC UACCCGUGGC AGGACGAAAA GACCCCGUGG AGCUUUACUG 60 T.thermo.
CUCGCUGUGA AGAUGCAGUG UACCCGCGGC AAGACGGAAA GACCCCGUGA ACCUUUACUA 2080 E.coli
CUACCUGUGA AGAUGCAGGU UACCCGCGAC AGGACGGAAA GACCCCGUGG AGCUUUACUG 2108 B.stearo.
CAGCCUGGUG UUGGCUCUUG GUCGCGCCUG CGUAGGAUAG GUGGGAGCCU GUGAACCCCC 120 T.thermo.
UAGCUUGACA CUGAACAUUG AGCCUUGAUG UGUAGGAUAG GUGGGAGGCU UUGAAGUGUG 2140 E.coli
CAGCCUGAUA UGGAAUUUUG GUGUCGCUUG UACAGGAUAG GUGGGAGCCU GGGAAGCCGG 2168 B.stearo.
GCCUCCGGGU GGGGGGGAGG CGCCGGUGAA AUACCACCCU GGCGCGGCUG GGGGCCUA-- 178 T.thermo.
GACGCCAGUC UGCAUGGAGC CGACCUUGAA AUACCACCCU UUAAUGUUUG AUGUUCUAAC 2200 E.coli
AGCGCCAGCU UCGGUGGAGG CGGCGGUGGG AUACCACCCU GGCGAUAUUG AAAUUCUAAC 2228 B.stearo.
----ACCC-- --UCGGAUGG GGGGACAGCG CUUGGCGGGC AGUUUGACUG GGGCGGUCGC 230 T.thermo.
GUUGACCCGU AAUCCGGGUU GCGGACAGUG UCUGGUGGGU AGUUUGACUG GGGCGGUCUC 2260 E.coli
CCGCACCCCU UAGCGGGGUG GGAGACAGUG UCAGGCGGGC AGUUUGACUG GGGCGGUCGC 2288 B.stearo.
CUCCUAAAAG -GUAACGGAG GCGCCCAAAG GUCCCCUCAG GCGGGACGGA AAUCCGCCGG 289 T.thermo.
CUCCU-AAAG AGUAACGGAG GAGCACGAAG GUUGGCUAAU CCUGGUCGGA CAUCAGGAGG 2319 E.coli
CUCCCAAAAG -GUAACGGAG GCGCCCAAAG GUUCCCUCAG AAUGGUUGGA AAUCAUUCGG 2347 B.stearo.
AGAGCGCAAG GGUAGAAGGG GGCCUGACUG CGAGGCCUGC AAGCCGAGCA GGGGCGAAAG 349 T.thermo.
UUAGUGCAAU GGCAUAAGCC AGCUUGACUG CGAGCGUGAC GGCGCGAGCA GGUGCGAAAG 2379 E.coli
AGAGUGCAAA GGCACAAGGG AGCUUGACUG CGAGACGGAC AGGUCGAGCA GGGACGAAAG 2407 B.stearo.
CCGGGCCUAG UGAACCGGUG GUCCCGUGUG GAAGGGCCAU CGAUCAACGG AUAAAAGUUA 409 T.thermo.
CAGGUCAUAG UGAUCCGGUG GUUCUGAAUG GAAGGGCCAU CGCUCAACGG AUAAAAGGUA 2439 E.coli
UCGGGCUUAG UGAUCCGGUG GUUCCGCAUG GAAGGGCCAU CGCUCAACGG AUAAAAGCUA 2467 B.stearo.
CCCCGGGGAU AACAGGCUGA UCUCCCCCGA GCGUCCACAG CGGCGGGGAG GUUUGGCACC 469 T.thermo.
CUCCGGGGAU AACAGGCUGA UACCGCCCAA GAGUUCAUAU CGACGGCGGU GUUUGGCACC 2499 E.coli
CCCCGGGGAU AACAGGCUGA UCUCCCCCAA GAGUCCACAU CGACGGGGAG GUUUGGCACC 2527 B.stearo.
UCGAUGUCGG CUCGUCGCAU CCUGGGGCUG AAGAAGGUCC CAAGGGUUGG GCUGUUCGCC 529 T.thermo.
UCGAUGUCGG CUCAUCACAU CCUGGGGCUG AAGUAGGUCC CAAGGGUAUG GCUGUUCGCC 2559 E.coli
UCGAUGUCGG CUCAUCGCAU CCUGGGGCUG UAGUCGGUCC CAAGGGUUGG GCUGUUCGCC 2587 B.stearo.
CAUUAAAGCG GCACGCGAGC UGGGUUCAGA ACGUCGUGAG ACAGUUCGGU CUCUAUCCGC 589 T.thermo.
AUUUAAAGUG GUACGCGAGC UGGGUUUAGA ACGUCGUGAG ACAGUUCGGU CCCUAUCUGC 2619 E.coli
CAUUAAAGCG GUACGCGAGC UGGGUUCAGA ACGUCGUGAG ACAGUUCGGU CCCUAUCCGU 2647 B.stearo.
CACGGGCGCA GGAGGCUUGA GGGGGGCUCU UCCUAGUACG AGAGGACCGG AAGGGACGCA 649 T.thermo.
CGUGGGCGCU GGAGAACUGA GGGGGGCUGC UCCUAGUACG AGAGGACCGG AGUGGACGCA 2679 E.coli
CGCGGGCGCA GGAAAUUUGA GAGGAGCUGU CCUUAGUACG AGAGGACCGG GAUGGACGCA 2707 B.stearo.
CCUCUGGUUU CCCAGCUGUC CCUCCAGGGG CAUAAGCUGG GUAGCCAUGU GCGGAAGGGA 709 T.thermo.
UCACUGGUGU UCGGGUUGUC AUGCCAAUGG CA-CUGCCCG GUAGCUAAAU GCGGAAGAGA 2738 E.coli
CCGCUGGUGU ACCAGUUGUC CCGCCAGGGG CA-CCGCUGG GUAGCUAUGU GCGGACGGGA 2766 B.stearo.
UAACCGCUGA AAGCAUCUAA GCGGGAAGCC CGCCCCAAGA UGAGGCCUCC CACGGCGUC- 769 T.thermo.
UAAGUGCUGA AAGCAUCUAA GCACGAAACU UGCCCCGAGA UGAGUUCUCC CUGACCCUUU 2798 E.coli
UAAGCGCUGA AAGCAUCUAA GCGUGAAGCC CCCCUCAAGA UGAGAUUUCC CACCGCGUC- 2826 B.stearo.
AAGCCGGU-- --AAGGACCC GGGAAGACCA CCCGGUGGAU GGGCCGGGGG UGUAAGCGCC 824 T.thermo.
AAG--GGUCC UGAAGGAACG UUGAAGACGA CGACGUUGAU AGGCCGGGUG UGUAAGCGCA 2856 E.coli
AGGCGGGU-- --AAGAUCCC UCGAAGAUGA CGAGGUCGAU AGGUCCGAGG UGGAAGCGUG 2881 B.stearo.
GCGAGGCGUU GAGCCGACCG GUCCCAAUCG UCCG--AGGU CUUGACCCCU C 873 T.thermo.
GCGAUGCGUU GAGCUAACCG GUACUAAUGA ACCGUGAGG- CUUAACC--U U 2904 E.coli
GCGACACGUG GAGCUGACGG AUACUAAUCG AUCG-AGGG- CUUAACC--U A 2928 B.stearo.

Table 1

Summary of data from analysis of the primary and secondary structure of 23 S rRNA region D from *T. thermophilus*

	Species	$^{3'}$G-C$^{5'}$	$^{3'}$C-G$^{5'}$	$^{3'}$G-U$^{5'}$	$^{3'}$U-G$^{5'}$	$^{3'}$A-U$^{5'}$	$^{3'}$U-A$^{5'}$
(A)	*E. coli*	28.2	26.3	7.9	7.5	15.8	14.3
	B. stearothermophilus	32.7	26.0	7.9	6.3	15.0	12.2
	T. thermophilus	38.1	33.1	7.8	4.7	9.7	6.6
		A	U	G	C	Σ	
(B)	*E. coli*	206/23.3	199/25.5	282/31.9	197/22.3	884	
	B. stearothermophilus	197/22.4	165/18.7	300/34.1	218/24.8	880	
	T. thermophilus	167/19.1	137/15.7	320/36.7	249/28.5	873	
		E. coli	*B. stearothermophilus*		*T. thermophilus*		
(C)	*E. coli*		E/B 76.81		E/T 74.21		
	B. stearothermophilus	B/E 77.16			B/T 81.14		
	T. thermophilus	T/E 75.14	T/B 81.79				

(A) Usage of basepairs in double-stranded regions; (B) usage of nucleotides expressed in real numbers and percent values; (C) percent homology by comparing all three sequences with each other, the values varying due to the different lengths of the sequences

$G^{5'}$, the occurrence of $^{3'}$G-U$^{5'}$ is comparable in *E. coli* as well as in *B. stearothermophilus* and *T. thermophilus* and does not reflect the considerable differences of their growth environments. Furthermore it is most remarkable that the use of $^{3'}$A-U$^{5'}$ and $^{3'}$U-A$^{5'}$ is, compared to *E. coli*, only slightly reduced in *B. stearothermophilus*, but drastically decreased in *T. thermophilus*.

Certain features of the large rRNA domain D (fig.3) are conserved, even when compared to eukaryotic rRNAs, i.e. the 12-nucleotide sequence containing the α-sarcin-cleavage site [13], which may contribute to the binding of the EF-1·GTP·aminoacyl-tRNA complex to the eukaryotic ribosome [14,15], and a region likely to be involved in the peptidyltransferase function. This region has been identified by sequencing yeast, mouse and human mitochondrial large-subunit rRNAs that confer chloramphenicol resistance [16,17] and by affinity labelling of *E. coli* 23 S rRNA with a reactive puromycin analogue [18]. Resistance reported so far was caused exclusively by nucleotide substitutions in this looped region [16,17].

Sites of mutations have been located at positions corresponding to residues 2447, 2451, 2452, 2503 and 2504 in *E. coli* [16,17,19–21]. In other experiments a photoreactive benzophenone derivative of yeast Phe-tRNA^{Phe} was employed to label *E. coli* ribosomes. The main site of modification was the uridine in position 2584, with some modification of the uridine in position 2585 [22]. Mutational changes in this region can lead to resistance to another antibiotic, erythromycin. Erythromycin is also a peptidyltransferase inhibitor, although its mode of action is quite different from that of chloramphenicol [23]. Erythromycin-resistant mutants of *E. coli* [24] and yeast mitochondria [25] have been isolated in which the exchange of a single base occurred at position 2058. This site is also found on the central loop in domain D (fig.4). Erythromycin resistance in *Staphylococcus* [26] has been shown to be due to dimethylation of adenine in a GAAAG (positions 2057–2061 in *E. coli*, fig.2) sequence. In *T. thermophilus* this sequence is somewhat different, namely AAAAG (positions 37–41 in *T. thermophilus*, fig.2), but exhibits three adenines at identical positions.

It was further established that the methylation

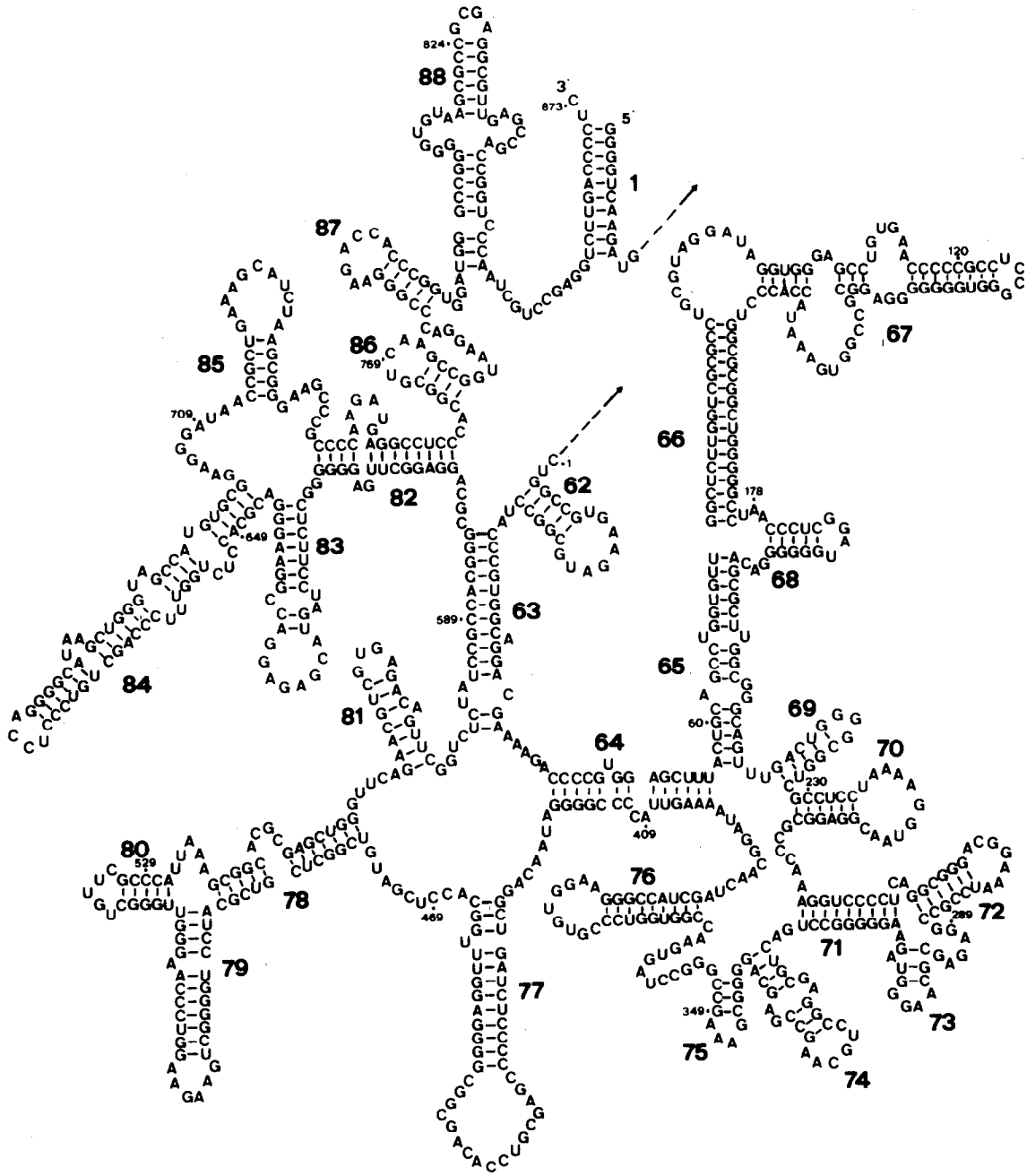

Fig.3. Secondary structure model for *T. thermophilus* HB8 23 S rRNA region D. The general model was derived from [33,34]. Nucleotides are numbered according to the presentation in table 1. Nucleotide 1 denotes nucleotide 2021 in the *E. coli* numbering and nucleotide 2149 in the *B. stearothermophilus* system.

of the adenine in position 2058 in eubacteria confers resistance against erythromycin [27]. Kethoxal-reactive sites in *E. coli* 23 S rRNA have been identified [28]. These sites are located at positions 2307, 2308, 2458 and 2470, i.e. positions 277, 278, 428 and 440, according to the arbitrary numbering applied to the *T. thermophilus* sequence (figs 2,3). In the secondary structure model

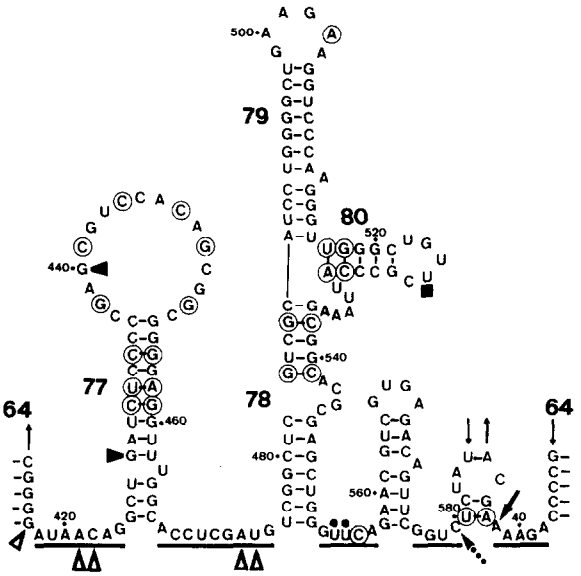

Fig.4. Conserved secondary structure of the peptidyltransferase region based on the sequence of *T. thermophilus* HB8 23 S rRNA. (Black square) Puromycin-reactive site in *E. coli* [18]; (black triangle) kethoxal-reactive site in *E. coli* [28]; (black dots) nucleotides which were shown to cross-link to a photoreactive Phe-tRNA^{Phe} in *E. coli* [22]; (open triangle) chloramphenicol-resistance site in eukaryotic mitochondria [16,17]; (arrow) erythromycin-resistance site in *E. coli* and yeast mitochondria [24,25,27]; (dotted arrow) a nucleotide, which was shown to be involved in an intramolecular tertiary interaction in *E. coli* [29]; (black line) nucleotides which represent the single-stranded region within the peptidyltransferase region; encircled nucleotides are different in the 23 S rRNA from *E. coli*.

these sites are all in proximity to the very highly conserved central loop in domain D of the 23 S rRNA. Tertiary interactions of regions in domain D with other regions in the 23 S rRNA have been determined in *E. coli* [29]. Cross-linked sites in the 23 S rRNA that represent potential tertiary contacts are 570/2030, 740/2610 and 1780/2570 [21]. The two positions located in domain D, equivalent to 2570 and 2610 in *E. coli*, are conserved in *T. thermophilus*. As a result of smaller deletions the stem structures 68, 73, 86 and 87 are shorter in *T. thermophilus* than in *E. coli*. A rather substantial number of base substitutions and compensating base exchanges occur in structures 65–68 and 71–74. The loops confined by helices 62, 80, 81, 83, and 85 are identical in all three species (figs 2,3). The three regions 66–68 (positions 2090–2200 in the *E. coli* numbering system) are believed to be involved in the binding of the ribosomal protein L1 [30]. The L1-binding site tends to be unusually rich in G-U pairs [31]. This is reflected in *T. thermophilus*, where the consecutively base paired helix 66 (fig.2) is composed of 11 G/C and 5 G/U pairs, but displays no A/U pairing, quite in contrast to 4 G/C, 7 A/U and 5 G/U pairs in the thermophilic Gram-positive eubacterium *B. stearothermophilus*. In *B. stearothermophilus* the occurrence of an unusual G-A base pair at the junction point of helices 65 and 66 (figs 2,3) was reported [12], which could not be found in *T. thermophilus*.

We analysed the DNA of *T. thermophilus* by employing thermal denaturation and CsCl density centrifugation. A high content of G/C (66.3%) could be found (not shown), which is comparable with *T. aquaticus* DNA, where 65.4% G/C could be determined [32]. This level of overall G/C content in the DNA is also reflected in domain D of the *T. thermophilus* 23 S rRNA (65.2%, table 1B).

In summarizing the present data we have found that the 23 S rRNA-region D from *T. thermophilus* HB8 differs, based on the usage of G/C basepairs (71.2%, table 1A) from the other eubacterial species, which could explain the degree of thermal stability of the rRNAs.

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