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The Sequence of the Single 16S rRNA Gene of the Thermophilic Eubacterium *Rhodothermus marinus* Reveals a Distant Relationship to the Group Containing *Flexibacter*, *Bacteroides*, and *Cytophaga* Species

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***Rhodothermus marinus*, a gram-negative heterotrophic marine thermophile, has been the subject of several recent studies. Isolation, sequencing, and analyses of a 16S rRNA gene have shown that *R. marinus* diverges sharply from major bacterial phyla and is most closely allied to the *Flexibacter-Cytophaga-Bacteroides* group. Further analyses revealed that the *R. marinus* chromosome contains a single rRNA operon with a 16S-23S intergenic region coding for $tRNA^{Ile}$ and $tRNA^{Ala}$.**

Thermophilic bacteria have been the subject of rising interest because of their biological adaptations and potential in biotechnology. A number of novel bacteria have been isolated at submarine hot springs and vents. Among these are eubacteria of the genus *Rhodothermus* which have been isolated in Iceland and the Azores (3, 15). *Rhodothermus marinus* is an aerobic heterotroph with optimum growth at 65 to 75°C (11) and may therefore be well suited for genetic engineering of thermostable enzymes. Plasmid vectors are being developed for this purpose (5), and genes coding for DNA ligase (23a), β-glucanase, and cellulase have been isolated (18a).

The genus *Rhodothermus* is not affiliated with the well-known thermophilic genus *Thermus* (19), and to clarify the phylogenetic status of the genus *Rhodothermus*, we isolated and sequenced a 16S rRNA gene and the 16S-23S intergenic spacer region coding for two tRNAs. Comparisons with other available 16S rRNA sequences utilizing several methods for reconstruction of phylogenies place the genus *Rhodothermus* close to the root of the *Flexibacter-Cytophaga-Bacteroides* (F-C-B) group with affinities to green sulfur bacteria, fibrobacteria, and spirochetes.

MATERIALS AND METHODS

DNA was extracted from *R. marinus* R-10 (DSM 4252, ATCC 43812) and used to construct a genomic library in phage EMBL4 (20). This library was screened with a ^{32}P-labeled probe made by reverse transcription of total *R. marinus* RNA primed with three universal 16S rRNA-specific oligonucleotides (13). DNA fragments generated from recombinant phage by restriction enzymes *Eco*RI and *Xho*I were cloned in phages M13mp18 and M13mp19 and screened with a 16S rRNA-specific probe as described above. Single-stranded M13 DNA was sequenced by the dideoxy method (21) by using modified T7 DNA polymerase (Sequenase; United States Biochemical) and a standard sequencing primer, 16S rRNA universal primers, or custom-made primers. DNA fragments generated by amplification with AmpliTaq (Perkin-Elmer) and labeled with digoxigenin (Boehringer Mannheim) were used as hybridization probes. 16S-23S intergenic sequences were amplified from bacterial DNA by using primers G1 and L1 (10). Inverse PCR of 16S 5′-terminal restriction fragments was performed by ligating 40 ng of digested bacterial DNA in 100 μl and then amplifying 1 ng in a 20-μl reaction volume by using divergent primers homologous to the 16S rRNA sequence (109R [CCC ACRYRTTACKCACCCGT] and 906F [GAAACTTAAAK GAATTG]). Aligned rRNA sequences were obtained from the Ribosomal Database Project (14).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number X80994.

RESULTS

A complete 16S rRNA gene from *R. marinus* was isolated in a recombinant EMBL4 phage. This cloned fragment of the bacterial chromosome contains approximately 0.5 kb upstream and 12 kb downstream of the 16S coding sequence. The entire sequence of this 16S rRNA gene and the 16S-23S intervening sequence coding for two tRNAs was determined via subcloning into M13 phage.

Previous work has shown that the genus *Rhodothermus* is distinct from the genus *Thermus* (3, 17, 19) but has given no indications of affinities to other bacterial genera. Inspection of the *R. marinus* 16S rRNA data revealed three sequence signatures characteristic of the F-C-B group and three that are also characteristic of the *Chlorobium* green sulfur bacteria (Table 1). At signature position 1410, there is a C which appears unique to the genus *Rhodothermus* and this also appears to be true for the adjacent G-A pairs at positions 1425 and 1426 (paired to 1474 and 1475) in the penultimate helix, where three G-A pairs are found in the F-C-B group (25). This helix is also longer, by five base pairs, than in most bacteria, a feature also seen in the genus *Aquifex* (4) and in eukaryotic 18S rRNA (16), supporting a deep phylogenetic branching of the genus *Rhodothermus*.

When the *R. marinus* 16S rRNA sequence is projected onto the secondary-structure model of *Escherichia coli* 16S rRNA (26), all major features are well conserved, substitutions being most common in the helixes, often with concerted replacement of the two bases in each pair. The *R. marinus* chromosome has a G+C content of 64% (3), and the 16S rRNA has a G+C content of 62%. In comparison, the *E. coli* chromosome contains 51% G+C (8) and the 16S rRNA is 54% G+C. This

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TABLE 1. Sequence signatures for the F-C-B group and relatives

Position(s)[a]	F-C-B group	*Thermonema lapsum*	*Rhodothermus marinus*	*Chlorobium* spp.	Others[b]
38	A	A	G	G	G
290–310	G-C	G-C	C-G	C-G	C-G
484	U or G	G	G	G	R
569–881	U-A	U-A	U-A	U-A	G-C
570	A	U	G	G	G
680–710	R-Y	G-C	C-G	C-G	C-G
724	U	A	G	G	G
866	A	A	C	C	C
975	G	G	G	A	A
995	A	A	A	A	C
1045	A, G, or U	A	G	C	C
1167	—[c]	—	—	—	A or U
1174.1	A, C, or U	—	G	A	C
1410	G	G	C	G	A
1475	A	A	A	G	G
1532	A	U	U	U	U

[a] Positions of bases or base pairs in the *E. coli* sequence (26).
[b] Consensus (>90%) of other bacteria (14, 25, 27).
[c] —, no nucleotide at this position.

is a reflection of a 16S rRNA structure, which is more strongly hydrogen bonded than that of *E. coli*, with approximately 65 new G-C pairs versus 30 bp changing from G-C to G-U or A-U.

When complete and aligned 16S rRNA sequences were compared, *R. marinus* did not show a high degree of similarity to any of the available aligned sequences (14). Even when areas of uncertain alignment were excluded and the comparison was restricted to purines versus pyrimidines, equal similarity to species from different bacterial phyla was found, indicating a deep branching and that distance matrix methods would not be effective in resolving the *R. marinus* phylogeny. Therefore, three different methods were employed to clarify the phylogenetic status of *R. marinus*.

Maximum-parsimony analysis (22) with complete 16S rRNA sequences from 30 representatives of all major bacterial groups (Table 2) yielded trees placing *R. marinus* close to the F-C-B group and green sulfur bacteria. To test the robustness of this placement, 17 sequences likely to be close to the *R. marinus* sequence were added and bootstrap analyses were run with various numbers of taxa. Possible bias was reduced by excluding positions of uncertain alignment (positions 69 to 100, 154 to 217, 452 to 484, 837 to 849, and 1441 to 1463 [*E. coli* numbering]), and bias due to selection for high G+C content was countered by considering only transversions (purines ⇔ pyrimidines). This analysis firmly placed *R. marinus* with the F-C-B group, as illustrated by the subset in Fig. 1.

Next, Lake's method of linear invariants (12) was utilized to test whether *R. marinus* is closer to the F-C-B group than to *Chlorobium* green sulfur bacteria, to spirochetes and fibrobacteria, or to purple bacteria. In all cases, the association of *R. marinus* with the F-C-B group was favored (Table 3). Also, transversion parsimony supports such grouping strongly, in accordance with the bootstrap analysis presented in Fig. 1. The parsimony tests also place the genus *Rhodothermus* closer to the green sulfur bacteria than to the spirochetes and fibrobacteria.

Maximum-likelihood analysis (7, 18) utilizing 16S rRNA sequences from *R. marinus* and various numbers of sequences also used in the parsimony analyses was used to construct phylogenetic trees. The results were consistent with the placement of *R. marinus* between the F-C-B group and the green sulfur bacteria (Fig. 2).

The number of 16S rRNA genes in *R. marinus* was determined as follows. The chromosomal DNA was digested with six different restriction enzymes, and the separated fragments were probed with labeled DNA corresponding to the 5′ third of the 16S rRNA molecule. In each case, a single restriction fragment was detected (Fig. 3), indicating a single rRNA operon in the *R. marinus* chromosome. This conclusion was supported by two different approaches. First, *R. marinus* chromosomal DNA was digested with the restriction enzyme *Bsa*HI and the digest was diluted and ligated to circularize the restriction fragments. Primers directing divergent DNA synthesis from sites internal to the 16S rRNA gene were used to amplify DNA from such circular self-ligated molecules. A single DNA product of the expected size (1,000 bp) was produced. Second, primers hybridizing to conserved 16S and 23S sequences were used to amplify the intergenic region (10). A single product of approximately 500 bp was produced. Sequencing confirmed the size of the 16S-23S intergenic space and revealed coding sequences for both $tRNA^{Ile}$ and $tRNA^{Ala}$ as found in the *rrnA*, *rrnD*, and *rrnH* operons of *E. coli* (15).

DISCUSSION

The capacity to grow at high temperatures is found in many different phylogenetic groups of bacteria, and many of the thermophiles branch deeply from the phylogenetic tree, indicating that thermophily is an ancient and perhaps original trait (1). The heterotrophic marine thermophile *R. marinus* was originally isolated at a single remote site in Iceland but has since been found in other locations (18b), as well as in the Azores (17). It may well be that the genus *Rhodothermus* and related bacteria yet to be discovered are widespread in the marine habitat, and it should not be surprising, considering that *R. marinus* is related to ubiquitous marine bacteria of the F-C-B group. As is true of many marine heterotrophs, *R. marinus* excretes a number of exoenzymes, many with biotechnological potential which is being pursued through cloning and expression in *E. coli* (18a).

Rapidly growing bacteria may have 6 to 10 rRNA operons (9, 15, 24). In contrast, the hyperthermophilic *Archaea* have only one rRNA operon (2). As shown here, *R. marinus* has only one rRNA operon sufficing for a 65°C growth temperature and an 80-min generation time (3). Coding regions for certain

TABLE 2. Aligned 16S rRNA sequences used in this study

RDP Short-ID[a] result	Organism	Group[a]
Aqu.pyroph	*Aquifex pyrophilus* Kol5A	2.1 Used as outgroup
Tt.maritim	*Thermotoga maritima* MSB8 (DSM 3109)	2.2 Order *Thermotogales*
		2.4 Green nonsulfur bacteria and relatives
Tmc.roseum	*Thermomicrobium roseum* (ATCC 27502)	2.4.1 *Chloroflexus* subdivision
Cfx.aurant	*Chloroflexus aurantiacus* J-10-fl (ATCC 29366)	2.4.1 *Chloroflexus* subdivision
D.radiodur	*Deinococcus radiodurans* (ATCC 35073)	2.4.2 *Deinococcus-Thermus* subdivision
T.thermoph	*Thermus thermophilus* HB8 (ATCC 27634)	2.4.2 *Deinococcus-Thermus* subdivision
		2.6 F-C-B
Anf.mariti	*Anaeroflexus maritimus* PL12FS (DSM 2831)	2.6.1 F-C-B subdivision I
Bac.fragil	*Bacteroides fragilis* (ATCC 25285)	2.6.1 F-C-B subdivision I
F.breve	*Flavobacterium breve* (ATCC 14234)	2.6.1 F-C-B subdivision I
Cy.lytica	*Cytophaga lytica* LIM-21 (ATCC 23178)	2.6.1 F-C-B subdivision I
Flx.canada	*Flexibacter canadensis* (ATCC 29591)	2.6.2 F-C-B subdivision II
Sap.grandi	*Saprospira grandis* (ATCC 23119)	2.6.2 F-C-B subdivision II
Flx.sancti	*Flexibacter sancti* (ATCC 23092)	2.6.2 F-C-B subdivision II
Cy.hutchin	*Cytophaga hutchinsonii* (ATCC 33406)	2.6.2 F-C-B subdivision II
Tnm.lapsum	*Thermonema lapsum*	2.6.2 F-C-B subdivision II
Rht.marinu	*Rhodothermus marinus* (DSM 4252)	2.6.2 F-C-B subdivision II
Chl.vibrio	*Chlorobium vibrioforme* 6030 (DSM 260)	2.7 Green sulfur bacteria
Chl.tepidu	*Chlorobium tepidum* NZC	2.7 Green sulfur bacteria
Chl.limico	*Chlorobium limicola* 8327	2.7 Green sulfur bacteria
Clt.sulfur	*Clathrochloris sulfurica* 1	2.7 Green sulfur bacteria
env.OS-9	Octopus Spring microbial mat DNA clone OSIII-9	2.7 Green sulfur bacteria
env.OS_M	Octopus Spring microbial mat clone OS Type M	2.7 Green sulfur bacteria
		2.9 *Planctomyces* spp. and relatives
Pln.staley	*Planctomyces staleyi* (ATCC 27377)	2.9.1 *Planctomyces* subdivision
Clm.psitta	*Chlamydia psittaci* 6 BC (ATCC VR 125)	2.9.2 *Chlamydia* subdivision
Fib.sucS85	*Fibrobacter succinogenes* S85 (ATCC 19169)	2.12 Fibrobacteria
Fib.intNR9	*Fibrobacter intestinalis* NR9 (ATCC 43854)	2.12 Fibrobacteria
		2.13 Spirochetes and relatives
Srp.hyodys	*Serpula hyodysenteriae* B256 (ATCC 31212)	2.13.1 Serpulina group
Spi.haloph	*Spirochaeta halophila* RS1 (ATCC 29478)	2.13.1 Serpulina group
Spi.stenos	*Spirochaeta stenostrepta* Z1 (ATCC 25083)	2.13.1 Serpulina group
Trp.pallid	*Treponema pallidum* Nichols	2.13.1 Serpulina group
Lpn.illini	*Leptonema illini* 3055	2.13.2 *Leptospira* group
		2.14 Purple bacteria
R.rubrum	*Rhodospirillum rubrum* ATH (ATCC 11170)	2.14.1 α subdivision
Ric.prowaz	*Rickettsia prowazekii* Breinl (ATCC VR 142)	2.14.1 α subdivision
Ag.tumefac	*Agrobacterium tumefaciens* (ATCC 4720)	2.14.1 α subdivision
Nis.gonor1	*Neisseria gonorrhoeae* (NCTC 8375)	2.14.2 β subdivision
Spr.voluta	*Spirillum volutans* (ATCC 19554)	2.14.2 β subdivision
Rcy.purpur	*Rhodocyclus purpureus* 6770 (DSM 168)	2.14.2 β subdivision
Chr.vinosm	*Chromatium vinosum* (ATCC 17899)	2.14.3 γ subdivision
E.coli	*Escherichia coli*	2.14.3 γ subdivision
Dsv.desulf	*Desulfovibrio desulfuricans* (ATCC 27774)	2.14.4 δ subdivision
Myx.xanthu	*Myxococcus xanthus* DK1622	2.14.4 δ subdivision
Wln.succi2	*Wolinella succinogenes* (ATCC 29543)	2.14.5 ε subdivision
Fus.nuclea	*Fusobacterium nucleatum* (ATCC 25586)	2.15 Fusobacteria and relatives
		2.16 Gram-positive phylum
Bif.breve	*Bifidobacterium breve* (ATCC 15700)	2.16.1 High-G+C subdivision
Arb.globif	*Arthrobacter globiformis* (DSM 20124)	2.16.1 High-G+C subdivision
C.pasteuri	*Clostridium pasteurianum* (ATCC 6013)	2.16.2 Clostridia and relatives
Hel.chlorm	*Heliobacterium chlorum* (ATCC 35205)	2.16.2 Clostridia and relatives
B.subtilis	*Bacillus subtilis*	2.16.5 Bacilli and relatives

[a] Ribosomal Data Project (RDP) version 4.0 short-ID labels and group numbers are shown (14).

tRNAs are commonly associated with the rRNA operons of bacteria. Thus, a $tRNA^{Ala}$ sequence intervening between the 16S and 23S regions appears to be widespread, e.g., in the hyperthermophilic *Archaea* (2) and in chloroplasts (23). In bacteria, the $tRNA^{Ala}$ sequence is usually preceded by a sequence coding for $tRNA^{Ile}$ (6). *Rhodothermus* species encode both of these tRNAs within a single rRNA operon.

The results of the bootstrap transversion parsimony analysis presented here firmly place the genus *Rhodothermus* at the root of the F-C-B group, but this result is sensitive to such

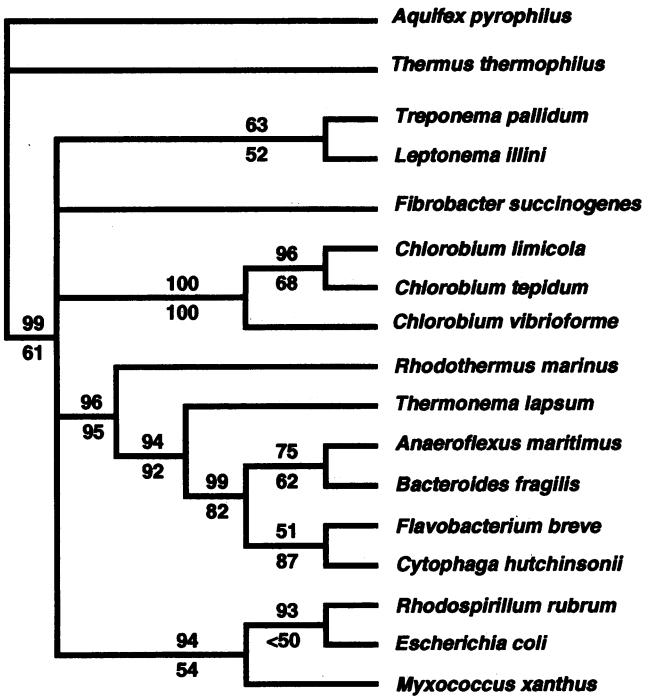

FIG. 1. Bootstrap maximum-parsimony analysis (22) of selected 16S rRNA sequences (1,000 replicates). Gaps and positions with uncertain alignment were excluded. Numbers indicate percentages of replicates yielding the grouping shown; numbers below lines are based on transversions only.

changes as allowing transitions or using only half of the 16S rRNA sequence. As both Lake's method of invariants (evolutionary parsimony) (12) and Felsenstein's method of maximum-likelihood analysis (7) yield the same result, it can be concluded that the genus *Rhodothermus* occupies a deeply branching new niche in the rapidly expanding tree of bacterial phylogenies and will serve as an important reference in future studies. One example is the comparison of nucleotide sequences, especially of closely related mesophilic and thermophilic bacteria, which can yield information on features important for thermal stability of structural RNA molecules. Thus,

TABLE 3. Parsimony tests of alternative trees[a]

Quartet[b]	No. of times tree favored		
	Lake's invariants	Standard parsimony	Transversion parsimony
(A, B), (C, D)	370	562	648
(A, C), (B, D)	148	66	23
(A, D), (B, C)	175	65	22
(B, C), (D, E)	116	170	74
(B, D), (C, E)	168	58	5
(B, E), (C, D)	310	366	515
(A, B), (D, E)	185	290	235
(A, D), (B, E)	169	128	107
(A, D), (B, D)	108	44	120

[a] All possible quartets from four phylogenetic groups (see Table 2) were evaluated by using PAUP (22).

[b] Groups: A, Spirochaetes and fibrobacteria; B, purple bacteria; C, F-C-B group; D, *R. marinus*; E, green sulfur bacteria.

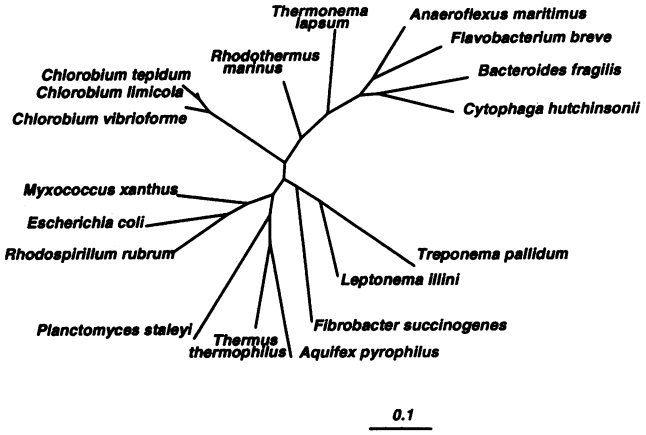

FIG. 2. Maximum-likelihood analysis of selected 16S rRNA sequences. Gaps and positions with uncertain alignment were excluded. The fastDNAml program (18) was used to determine the most likely tree by randomly jumbling the taxon input order until the same most-likely tree was found five times. The bar indicates the expected number of nucleotide substitutions per site.

the general trend of increasing G-C pairs is clear in comparing the 16S rRNA secondary structures of the mesophile *E. coli* and the thermophile *R. marinus*. A parallel increase in G-C base pairs is seen in the $tRNA^{Ile}$ from *R. marinus* and *Thermus thermophilus* compared with *E. coli*, so that the *R. marinus* tRNA appears to be more related to the *T. thermophilus* tRNA than to the tRNA from its closer relative. In addition to furthering understanding of structure and stability, increased information on stable RNA sequences from a variety of psychro-, meso-, and thermophiles can give clues to the question of whether thermophily is an ancestral state among bacteria and thus shed light on the origin and evolution of life (1).

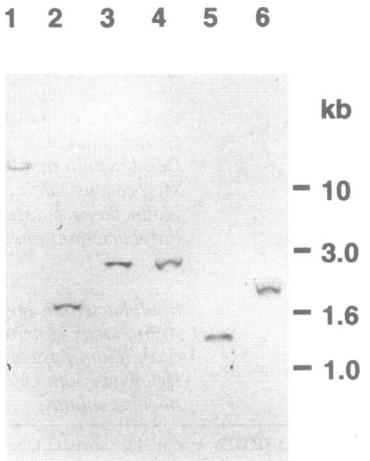

FIG. 3. Hybridization analysis of the *R. marinus* genome to determine the number of rRNA operons. *R. marinus* DNA was digested with various restriction enzymes, electrophoresed through 0.8% agarose, and transferred to a Hybond N^+ membrane (Amersham). The blot was probed with a labeled PCR product corresponding to positions 43 through 519 in the 16S rRNA. Lanes: 1, *Eco*RI; 2, *Hin*cII; 3, *Nco*I; 4, *Pst*I; 5, *Sma*I; 6, *Bsa*HI.

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