Reevaluation of the Phylogenetic Depth of the Marine Aerobic Hyperthermophilic Archaeon *Aeropyrum pernix* Based on Comparative Analysis of 23S rRNA Sequences

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Aeropyrum pernix, a strictly aerobic marine hyperthermophilic archaeon, has been studied as a potential biotechnological source. In order to clarify the phylogenetic position of *A. pernix*, the nearly complete nucleotide sequence of the 23S rRNA was determined and compared with the other hitherto known archaeal counterparts. To avoid the artifact caused by disparities of G + C content, transversion evolutionary distance analysis was adopted. The result confirmed that *A. pernix* is closely allied to the *Pyrodictium-Desulfurococcus-Staphylothermus* group (the order “*Igneococcales*”) within the kingdom *Crenarchaeota*. However, it also revealed that the genus *Aeropyrum* diverges sharply from the other genera within the family *Desulfurococcaceae*. Thus, we concluded that the decision to transfer the genus *Aeropyrum* from the family *Desulfurococcaceae* is reasonable in light of our present data, as well as the physiological characteristics of this archaeon reported previously.

Key words: *Aeropyrum pernix*, molecular phylogeny, archaea, *Crenarchaeota*, 23S rRNA, hyperthermophile, transversion evolutionary distance

Marine hyperthermophilic archaea (formerly called archaebacteria) have potential biotechnological and commercial significance. They have provided a lot of valuable resources for exploitation in novel industrial processes and in developing our understanding of how biomolecules are stabilized when under extremely high temperatures. Over the past fifteen years, a number of novel archaea have been isolated from marine hydrothermal environments, such as submarine hot springs and vents.^{1,2)} We have also isolated a novel archaeon *Aeropyrum pernix* at a coastal solfataric vent in Kodakara Island, Japan.^{3,4)} *A. pernix* is a strictly aerobic heterotroph growing optimally at 90 to 95°C and may therefore be well suited for biotechnological application of thermostable enzymes. Various types of proteinases are being developed for this purpose,^{5,6)} and genes for rRNA intron-encoded homing endonucleases^{7,*} and DNA polymerase (Ishino *et al.*, Personal communication) have been isolated from this organism.

Our previous study suggested that *A. pernix* is a distinctive lineage related to the genera *Pyrodictium* and *Desulfurococcus* within the kingdom *Crenarchaeota*.³⁾ However, the valid phylogenetic position of *A. pernix* was difficult to determine, since reconstitution of the neighbor-joining phylogenetic tree based on 16S rRNA sequences were ambiguous. Moreover, only a limited number of sequences were used for the analysis due to variation of the biased base composition. Subsequently, Burggraf *et al.* reported that the most probable 16S rRNA-based phylogenetic trees determined by most algorithms place the genus *Aeropyrum* in the family *Desulfurococcaceae*,

although occasionally some algorithms place this genus in the family *Pyrodictiaceae*.⁸⁾ These results suggest that genetic information of 16S rRNA is not enough to determine the exact and robust position of *A. pernix*.

23S rRNA shares the features as a molecular clock with 16S rRNA and has the added advantages of an even longer chain length, which might provide more reliable information for inferring phylogenies.⁹⁾ Therefore, we determined the nearly complete 23S rRNA sequence of *A. pernix* and resolved the phylogeny of the genus *Aeropyrum*.

Materials and Methods

Cultivation, Preparation of 23S rRNA, and Sequencing

Aeropyrum pernix (strain K1; JCM 9820) cells were grown in JXT medium at 90°C with vigorous shaking and harvested in the late exponential phase.³⁾ 23S rRNA was extracted and purified as previously reported.⁷⁾ The cDNA clone of the 23S rRNA was obtained after reverse transcription and PCR amplification. The oligonucleotide (DmLR3070R, 5'-CGGGCTCTTGGGAGCGGCG-3'), complementary to the 3'-tail region of the 23S rRNA of *Desulfurococcus mobilis*¹⁰⁾ was used to prime first-strand synthesis with Superscript II RNaseH⁻ (GIBCO Laboratories, Grand Island, N.Y.). The reverse transcription reaction was performed according to the manufacture's instructions, and the product was amplified by PCR with 0227aF (5'-GAGGAAAAGAAATCAA-3')¹¹⁾ and DmLR3070R as primers. Thirty five amplification cycles of 90 s at 96°C, 1 min at 62°C, two minutes at 72°C were

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performed. The double-stranded PCR product was cloned directly into the pCRII vector (Invitrogen, San Diego, Ca.) to yield plasmid pNB23. Deletion mutants were produced by progressive exonuclease III digestion,¹²⁾ and the nucleotide sequences were determined by the dideoxy sequencing method.¹³⁾

Alignment and Phylogenetic Methods

The *A. pernix* 23S rRNA sequence was aligned using the CLUSTAL W,¹⁴⁾ to the sequences previously described for 24 Archaea and 1 Bacterium available from the DDBJ, EMBL, and GenBank nucleotide sequence databases and from LSU rRNA database¹⁵⁾ and checked manually. Nucleotides undetermined for any of the sequences and regions of uncertain alignment were omitted from the analysis and evolutionary distances were calculated for 1,242 nucleotides from position 1,261 to 2,788 (*E. coli* numbering system¹⁶⁾) using the correction of Kimura's 2-parameter model.¹⁷⁾ The phylogenetic tree was constructed by the neighbor-joining method¹⁸⁾ from the transversion evolutionary distance matrix.¹⁹⁾ Bootstrap analysis²⁰⁾ was used to generate confidence estimates for tree topologies.

Nucleotide Sequence Accession Number

The 23S rRNA sequence of *A. pernix* will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB004787.

Results

The partial 23S rRNA sequence of *A. pernix*, from positions 228 to 2871 (according to *E. coli* numbering) representing 2842 nucleotides, was determined. The G+C

content of this sequence was 69.0 mol%.

The sequences employed in the comparative analysis had G+C contents in the range of 51.8 to 67.6 mol%, while purine contents ranged from 55.1 to 56.6 mol%. Therefore, to avoid the artifact caused by compositional disparities, transversion evolutionary distances were estimated (Table 1), and a phylogenetic tree was constructed by the neighbor-joining method (Fig. 1). Approximate topology of the tree was similar to the reported one based on 16S rRNA²¹⁾ with the exception of the convergence between the order *Thermococcales* and the cluster of the orders *Methanococcales*-*Methanobacteriales*. According to the phylogenetic tree, the kingdom *Crenarchaeota* is composed of three major branches corresponding to three orders, *Thermoproteales*, *Sulfolobales*, "Igneococcales". The tree showed that *A. pernix* was a deeply branching representative, which was located above the order *Sulfolobales* and below branching the family *Pyrodictiaceae* from the family *Desulfurococcaceae* within the kingdom *Crenarchaeota*. The bootstrap value of 100% among 1000 resampling supported the grouping of the genus *Aeropyrum*, the family *Pyrodictiaceae*, and the family *Desulfurococcaceae*. Besides, the bootstrap value for the nodes branching the three species *Pyrodictium occultum*, *Desulfurococcus mobilis*, and *Staphylothermus marinus* were relatively high (85% and 87%), indicating the probability of their branching order. Particularly noteworthy was that *A. pernix* represented the second shortest, therefore primitive, lineage in this archaeal tree.

A. pernix 23S rRNA shared a secondary structural feature with the families *Pyrodictiaceae* and *Desulfurococcaceae* that might be a defining signature of this particular group (Fig. 2). The helix at positions 1445 to 1465 in the 23S rRNA secondary structure (*E. coli* numbering) of all

Fig. 1. Phylogenetic relationships for the crenarchaea and the position of *Aeropyrum pernix* with 23S rRNA transversion evolutionary distances of Table 1.

Bar represents 5 transversional nucleotide substitution per 100 site. The sequence of the bacterium *Thermotoga maritima* was used as an out-group. Numbers at nodes represent bootstrap values for that node based on 1000 bootstrap resamplings.

Table 1. Nucleotide ratios and transversion evolutionary distances among representative 25 archaeal and 1 bacterial 23S rRNA sequences

Species	G+C mol%	A+G mol%	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1 <i>Aeropyrum pernix</i>	67.6	55.6	—																									
2 <i>Pyrodicticum occultum</i>	67.1	55.9	0.0314	—																								
3 <i>Desulfurococcus mobilis</i>	65.6	55.5	0.0338	0.0314	—																							
4 <i>Staphylothermus marinus</i>	66.3	56.2	0.0346	0.0242	0.0250	—																						
5 <i>Acidianus brierleyi</i>	61.9	56.2	0.0701	0.0741	0.0733	0.0709	—																					
6 <i>Acidianus infernus</i>	63.0	56.3	0.0693	0.0749	0.0693	0.0701	0.0073	—																				
7 <i>Sulfolobus acidocaldarius</i>	62.4	56.1	0.0725	0.0701	0.0741	0.0733	0.0266	0.0258	—																			
8 <i>Sulfolobus shibatae</i>	63.0	56.2	0.0766	0.0693	0.0717	0.0725	0.0274	0.0282	0.0266	—																		
9 <i>Sulfolobus solfataricus</i>	63.0	56.3	0.0757	0.0685	0.0709	0.0717	0.0282	0.0290	0.0274	0.0024	—																	
10 <i>Stygiolobus azoricus</i>	62.9	56.2	0.0717	0.0693	0.0717	0.0709	0.0258	0.0234	0.0121	0.0242	0.0250	—																
11 <i>Thermofilum pendens</i>	65.0	56.4	0.0774	0.0766	0.0790	0.0798	0.0878	0.0870	0.0919	0.0975	0.0967	0.0878	—															
12 <i>Thermoproteus tenax</i>	65.1	55.9	0.0717	0.0725	0.0749	0.0806	0.0951	0.0959	0.0975	0.0951	0.0943	0.0935	0.0508	—														
13 <i>Pyrobaculum islandicum</i>	65.9	56.3	0.0709	0.0733	0.0790	0.0798	0.0991	0.0983	0.0999	0.0975	0.0967	0.0959	0.0516	0.0105	—													
14 <i>Thermococcus celer</i>	63.3	55.9	0.0959	0.0983	0.1023	0.1080	0.1209	0.1185	0.1217	0.1225	0.1217	0.1209	0.0991	0.0951	0.0975	—												
15 <i>Pyrococcus furiosus</i>	64.3	55.8	0.0870	0.0943	0.0967	0.1039	0.1201	0.1176	0.1209	0.1249	0.1241	0.1185	0.1015	0.0959	0.0983	0.0137	—											
16 <i>Methanococcus vannielii</i>	57.2	55.9	0.1056	0.1048	0.1152	0.1144	0.1176	0.1201	0.1185	0.1257	0.1249	0.1209	0.1023	0.1064	0.1120	0.0774	0.0733	—										
17 <i>Methanobacterium thermoautotrophicum</i>	58.1	55.4	0.0959	0.1064	0.1088	0.1160	0.1176	0.1168	0.1135	0.1176	0.1168	0.1144	0.1039	0.1015	0.1039	0.0757	0.0749	0.0629	—									
18 <i>Thermoplasma acidophilum</i>	54.9	55.5	0.1571	0.1483	0.1620	0.1612	0.1628	0.1636	0.1555	0.1579	0.1571	0.1547	0.1475	0.1467	0.1475	0.1273	0.1297	0.1418	0.1305	—								
19 <i>Archaeoglobus fulgidus</i>	62.2	56.6	0.1305	0.1330	0.1370	0.1330	0.1346	0.1354	0.1338	0.1378	0.1370	0.1346	0.1338	0.1378	0.1386	0.1023	0.0999	0.0975	0.1104	0.1313	—							
20 <i>Methanospirillum hungatei</i>	51.8	55.9	0.1426	0.1402	0.1475	0.1434	0.1547	0.1555	0.1539	0.1563	0.1555	0.1547	0.1426	0.1467	0.1523	0.1225	0.1233	0.1176	0.1241	0.1467	0.1039	—						
21 <i>Halobacterium marismortui</i>	56.2	55.5	0.1459	0.1515	0.1555	0.1547	0.1595	0.1571	0.1620	0.1676	0.1668	0.1595	0.1394	0.1483	0.1491	0.1338	0.1346	0.1273	0.1241	0.1515	0.1168	0.1193	—					
22 <i>Haloferax volcanii</i>	55.7	55.4	0.1516	0.1589	0.1597	0.1589	0.1669	0.1645	0.1677	0.1734	0.1726	0.1669	0.1468	0.1540	0.1548	0.1411	0.1419	0.1250	0.1202	0.1637	0.1177	0.1234	0.0556	—				
23 <i>Halococcus morrhuae</i>	58.0	54.7	0.1483	0.1523	0.1579	0.1555	0.1636	0.1612	0.1644	0.1716	0.1708	0.1636	0.1418	0.1507	0.1515	0.1378	0.1386	0.1281	0.1265	0.1571	0.1144	0.1185	0.0363	0.0419	—			
24 <i>Natronobacterium magadii</i>	56.8	55.1	0.1660	0.1700	0.1757	0.1732	0.1829	0.1805	0.1869	0.1894	0.1886	0.1845	0.1612	0.1668	0.1676	0.1491	0.1499	0.1442	0.1362	0.1636	0.1241	0.1249	0.0459	0.0452	0.0403	—		
25 <i>Halobacterium halobium</i>	55.7	55.7	0.1539	0.1563	0.1652	0.1595	0.1644	0.1620	0.1652	0.1708	0.1700	0.1676	0.1410	0.1531	0.1523	0.1370	0.1378	0.1289	0.1176	0.1531	0.1072	0.1160	0.0403	0.0460	0.0346	0.0363	—	
26 <i>Thermotoga maritima</i>	62.7	56.6	0.1531	0.1555	0.1579	0.1587	0.1652	0.1644	0.1692	0.1716	0.1692	0.1684	0.1483	0.1555	0.1547	0.1523	0.1531	0.1684	0.1620	0.1910	0.1902	0.1797	0.2006	0.1984	0.1966	0.2111	0.1974	—

For nucleotide ratios, only positions used in the phylogenetic analysis were included in this calculation.

The sequences used in this calculations were obtained from DDBJ/EMBL/GenBank nucleotide sequence databases with the exception of the sequence of *Haloferax volcanii* and *Thermoproteus tenax*, which were extracted from LSU rRNA database.¹⁹ Nucleotide sequence accession numbers are as follows: *Pyrodicticum occultum*, M86626; *Desulfurococcus mobilis*, X05480; *Staphylothermus marinus*, M86623; *Acidianus brierleyi*, U32317; *Acidianus infernus*, U32318; *Sulfolobus acidocaldarius*, U05018; *Sulfolobus shibatae*, U32321; *Sulfolobus solfataricus*, U32322; *Stygiolobus azoricus*, U32319; *Thermofilum pendens*, X14835; *Pyrobaculum islandicum*, M86622; *Thermococcus celer*, M67497; *Pyrococcus furiosus*, M86627; *Methanococcus vannielii*, X02729; *Methanobacterium thermoautotrophicum*, X15364 and X05482; *Thermoplasma acidophilum*, M32298 and M20822; *Archaeoglobus fulgidus*, M64487; *Methanospirillum hungatei*, M81323 and M61738; *Halobacterium marismortui*, X13738; *Halococcus morrhuae*, X05481; *Natronobacterium magadii*, X72495; *Halobacterium halobium*, X03407, X00872, and X01699; *Thermotoga maritima*, M67498. A total of 1242 unambiguous nucleotides were used in the analysis.

Fig. 2. Diagnostic putative secondary structure of the 1445-to-1465 region (*E. coli* numbering) of several 23S rRNAs which distinguishes the members of the order "Igneococcales" from the other archaea.

sequences was characterized by a G bulge 6 nucleotides 5' from the capping loop and a AG bulge 6 nucleotides 3' from the capping loop. This motif was not found elsewhere in the domain *Archaea*.

Discussion

Burggraf *et al.* described that the genus *Aeropyrum* could be assigned to the family *Desulfurococcaceae* in accord to the 16S rRNA-based phylogenetic analyses.⁹⁾ However, the tree topology was considerably unstable, therefore, the above conclusion is not necessarily valid from a statistical point of view. Therefore, we reexamined the crenarchaeal phylogeny on the basis of 23S rRNA sequences and determined the relationship of the genus *Aeropyrum* to the genera in the family *Desulfurococcaceae* with whom it is currently grouped.

Due to the different base substitution rates of evolution in 23S rRNA sequences employed in this analysis (Table. 1), it is difficult to define specific phylogenetic distances separating classes of taxa. Sequences from hyperthermophiles have a high G+C content, which stabilizes the secondary structure at high temperatures. This results in a lower rate of base substitution than that observed for

mesophilic organisms such as extreme halophiles and methanogens. While the G+C content shows significant variation from one sequence to another, the purine (and therefore, pyrimidine) content of the sequences is nearly constant (Table. 1). Thus, we performed transversion distance analysis to correct the branching order artifacts produced by G+C content disparities.

Despite the clustering of the genus *Aeropyrum* and the genera in the families *Pyrodictiaceae* and *Desulfurococcaceae*, the genus *Aeropyrum* should not be assigned to the family *Desulfurococcaceae*, since (i) the transversion distance between *Aeropyrum pernix* and its neighbor *Pyrodictium occultum* (3.1 substitutions per 100 nucleotides) is as high as that between *P. occultum* and *Desulfurococcus mobilis* (3.1 substitutions), each of which belongs to different archaeal families, and (ii) the lack of a common physiological feature is a strong argument that the genus *Aeropyrum* belongs to neither the family *Pyrodictiaceae* nor *Desulfurococcaceae*. Growth of *A. pernix* is not observed under anaerobic conditions in the presence or absence of elemental sulfur, whereas all the members of the families *Pyrodictiaceae* and *Desulfurococcaceae* are unable to thrive aerobically.²²⁻²⁶⁾

The aerobic marine hyperthermophile *A. pernix* was

originally isolated at a single site in Japan but has since been found in several different geographical locations (Nomura *et al.*, Unpublished data). The genus *Aeropyrum* and its related organisms yet to be discovered might be widespread in the marine hydrothermal environments. Considering that *in situ* detection of the 16S rDNA fragment revealed the high abundance of marine planktonic crenarchaea in the ocean,^{27–30} this speculation should not be surprising. The establishment of the new family “*Aeropyraceae*” might be led after the isolation and detailed characterization of as-yet-undiscovered organisms closely related to the the genus *Aeropyrum*.

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