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Diversity of 16S rRNA gene, ITS region and *aclB* gene of the *Aquificales*

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Abstract The *Aquificales* are prevalent members of the microbial communities inhabiting many marine and terrestrial hydrothermal systems. Numerous new strains were obtained from deep-sea and terrestrial hydrothermal systems. In order to resolve the phylogenetic relationships within this group, three different phylogenetic datasets were used, namely the 16S rRNA gene, the intergenic transcribed spacer region between the 16S rRNA and 23S rRNA genes (ITS) and the gene coding for the ATP citrate lyase (*aclB*), a key enzyme in the reductive TCA cycle. The data were analyzed using neighbor-joining, parsimony and maximum likelihood. The resulting phylogenies appeared to be consistent between the three markers. The three genes confirmed the presence of isolates that merit further characterization and descriptions as new species and perhaps even new genera. The detailed phylogenetic interrelationships of these isolates are described here.

Keywords Thermophiles · Aquificales · 16S rRNA · ITS · *aclB*

Introduction

The *Aquificales* are a group of thermophilic, chemolithotrophic and microaerophilic bacteria that are widespread in hydrothermal systems. Members of this group have been isolated from shallow marine and deep-sea vents and from terrestrial hot springs. Initially, all members isolated belonged to the family *Aquificaceae* that presently includes the genera *Aquifex*, *Hydrogenobaculum*, *Hydrogenobacter*, *Thermocrinis*, and the recently described *Hydrogenivirga* (Kryukov et al. 1983; Kawasumi et al. 1984; Kristjansson et al. 1985; Nishihara et al. 1990; Huber et al. 1992, 1998; Shima and Suzuki 1993; Nakagawa et al. 2004). However, sequences belonging to a disparate lineage were found in clone libraries from environmental samples from deep-sea vents and terrestrial hot springs in Yellowstone National Park (YNP, Reysenbach et al. 2000a, b, 2005). The marine and terrestrial microorganisms represented by these latter sequences have been recently isolated and form a second family within the *Aquificales*, the *Hydrogenothermaceae* (*Persephonella*, *Hydrogenothermus*, *Sulfurihydrogenibium*) (Stöhr et al. 2001; Götz et al. 2002; Takai et al. 2003; Aguiar et al. 2004; Nakagawa et al. 2005). This second lineage appears to be widespread at high temperature, near neutral terrestrial hot springs. Environmental sequence analyses suggest that this group is very diverse and dominant (Yamamoto et al. 1998; Reysenbach et al. 2000a, 2002; Skirnisdottir et al. 2000; Spear et al. 2005). Moreover, a third lineage closely related to the *Aquificales*, namely the *Desulfurobacteriaceae*, has been found at deep-sea vents. However, it is still debatable whether this third group belongs to the *Aquificales*, or should be classified as a distinct order or family (L'Haridon et al. 1998, 2006; Huber et al. 2002).

Based on the analysis of the small subunit (16S) rRNA gene sequences, the *Aquificales* represent the deepest lineage within the bacterial domain (Burggraf et al. 1992; Pitulle et al. 1994). However, this placement remains controversial because of factors, such as high GC content bias. In contrast, genome comparisons, RNA polymerase sequence and signature protein sequences support a later divergence of the order *Aquificales* (Deckert et al. 1998; Bocchetta et al. 2000; Griffiths and Gupta 2004). Although the use of 16S rRNA gene is very useful in studies on microbial diversity, it has some pitfalls, in particular poorly resolving some close relationships, for example, species of the same genus

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(Konstantinidis and Tiedje 2005). On the contrary, the spacer region located between 16S and 23S rRNA genes, is in general extremely variable in size and sequence even within closely related species (Gurtler and Stanisich 1996) and has been used to resolve phylogenetic relationships among many groups such as the ammonia-oxidizing bacteria (Aakra et al. 2001) and some *Alpha-proteobacteria* (Kwon et al. 2005). In cyanobacteria, the intergenic transcribed spacer (ITS) region has been used successfully to differentiate closely related strains (Rocap et al. 2002; Ferris et al. 2003; Janse et al. 2004) but also to determine wider biogeographical patterns in cyanobacteria inhabiting hot springs from different geographical regions (Papke et al. 2003). Furthermore, protein-coding genes have recently been used to infer phylogenetic relationships within different groups of organisms, like ammonia-oxidizing (*amoA*) (Purkhold et al. 2000), methane-oxidizing (*pmoA*, *mxaF* and *moX*) (Heyer et al. 2002) and mercury-reducing bacteria (*merA*) (Vetriani et al. 2005).

Due to their controversial position in the universal tree of life, much effort has focused on studying the phylogenetic placement of the *Aquificales* relative to other major groups of Bacteria and Archaea. However, little is known about the phylogenetic interrelationships of the *Aquificales*. Here, we have explored the phylogenetic diversity of multiple new strains of *Aquificales* from deep-sea vents in the Mid-Atlantic Ridge (MAR), the Central Indian Ridge (CIR) and the East Pacific Rise (EPR), and from hot springs in Chile, Costa Rica, Kamchatka and YNP. We have used the 16S rRNA gene, ITS region and a gene coding for a key enzyme in the reductive TCA cycle, the β-ATP citrate lyase (*aclB*), to expand our perspective of the phylogenetic relationships of the *Aquificales*. We also evaluate the utility of each region to distinguish different hierarchical levels, ranging from closely related strains to different genera, and assess the suitability of these genes for the phylogenetic analysis of the *Aquificales*.

Methods

Isolation and growth conditions

Deep-sea vent cultures of *Aquificales* were isolated from high-temperature sulfide rock slurries as described by Götz et al. (2002), and terrestrial *Aquificales* strains were isolated from hot spring samples as described by Aguiar et al. (2004) (see Table 1 for collection information). All strains were isolated with either sulfur and oxygen or hydrogen and oxygen as electron donors and acceptors, respectively. Other strains were obtained from the OCM culture collection (Oregon, USA; methanogens.pdx.edu) or DSMZ (Germany; www.dsmz.de). We were unable to obtain viable cultures or DNA of *Hydrogenothermus marinus* and *Hydrogenobaculum acidophilum* from DSMZ or other sources.

DNA extraction and PCR conditions

DNA was extracted from cell pellets by using a standard extraction kit (DNeasy Tissue Kit; Qiagen, Valencia, CA, USA) following the manufacture's protocol. Extracted DNA was stored in 10 mM Tris at −20°C.

16S rRNA genes were amplified by PCR using the primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The ITS region was amplified using the forward primer 1492F targeting the end of 16S rRNA gene (5′-AAGTCGTAACAAGGTAACC-3′) and the reverse primer 115R (5′-GGGTTBCCCCATTCRG-3′) that targeted the 23S rRNA gene (García-Martínez et al. 1999). Both primers are specific for Bacteria. For β-ATP citrate lyase gene amplification, the degenerate primers described by Campbell et al. (2003), 892F (5′-TGGAC-MATGGTDGCYGGKGGT-3′) and 1204R (5′-ATA-GTTKGGSCCACCTCTTC-3′) were used.

For amplification of 16S rRNA gene and ITS region each reaction contained: DNA template (1–10 ng), dNTPs (200 μM each), $MgCl_2$ (1.5 mM), primers (0.4 μM each), *Taq* DNA polymerase (1 U) and the PCR buffer supplied by the manufacturer (Promega, Madison, WI, USA). For the *aclB* gene amplification, all the reagent conditions were the same with the exception of $MgCl_2$ that was increased in concentration from 1.5 to 3 mM. PCR conditions for amplification of the 16S rRNA gene were as described in Takacs et al. (2001). PCR conditions for amplifying ITS region were as follows: initial cycle of 94°C for 5 min, 30 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 1 min, and a final extension for 5 min at 72°C. Reactions for amplifying *aclB* gene were as described in Campbell et al. (2003). However, for *aclB* amplification in some cases the annealing temperature was varied through successive PCR reactions from 53 to 65°C in order to eliminate nonspecific amplification. PCR products were purified with the Ultraclean PCR Clean-up Kit (MoBio, Carlsbad, CA, USA) and stored in 10 mM Tris at −20°C.

Sequencing and phylogenetic analysis

PCR products were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit and an ABI 310 Genetic Analyzer according to the manufacturer's protocol (Applied Biosystems Inc., Foster City, CA, USA). For each new isolate, the complete sequence of both strands of 16S rRNA gene was obtained using a suite of 16S rRNA gene-specific primers to generate an overlapping set of sequences that were assembled into one contiguous sequence using AutoAssembler (Applied Biosystems Inc.). Because of the short length of the fragments for the ITS region and *aclB* gene, sequencing reactions were performed only with the same set of primers used for PCR amplification, and both strands were sequenced.

Table 1 Strains of *Aquificales* used in this study and their site of isolation

Strain	Site of isolation	*T* (°C)/pH	Reference
Hydrogenothermaceae			
Persephonella marina[a]	East Pacific Rise, DSV	70/6	Götz et al. (2002)
Persephonella guaymasensis[a]	Guaymas Basin, DSV	75/6	Götz et al. (2002)
Persephonella hydrogeniphila	Izu-Bonin Arc, Japan, DSV	70/7.2	Nakagawa et al. (2003)
Persephonella str. MAR9703[a]	Mid-Atlantic Ridge, DSV	70/6	Reysenbach et al. (2002)
Persephonella str. MAR10202	Mid-Atlantic Ridge, DSV	70/6	Reysenbach et al. (2002)
Persephonella str. CIR2951[a]	Central Indian Ridge, DSV	70/6	This study
Persephonella str. CIR2971[a]	Central Indian Ridge, DSV	70/6	This study
Persephonella str. CIR297H[a]	Central Indian Ridge, DSV	70/6	This study
Persephonella str. EPR351[a]	East Pacific Rise, DSV	70/6.5	This study
Persephonella str. EPR486[a]	East Pacific Rise, DSV	70/6.5	This study
Hydrogenothermus marinus	Marine Spring, Vulcano, Italy	65/3–4	Stöhr et al. (2001)
Sulfurihydrogenibium yellowstonense[a]	Calcite Springs, YNP, HS	70/7.5	Nakagawa et al. (2005)
Sulfurihydrogenibium azorense[a]	Azores Islands, HS	68/6	Aguiar et al. (2004)
Sulfurihydrogenibium subterraneum[a]	Subsurface aquifer, Japan	60–65/7.5	Takai et al. (2003)
Sulfurihydrogenibium str. 153IV9[a]	El Tatio, Chile, HS	60/6.3	This study
Sulfurihydrogenibium str. Y04ANG1[a]	Mammoth Springs, YNP, HS	70/6.3	This study
Sulfurihydrogenibium str. Y04ACS1[a]	Calcite Springs, YNP, HS	70/6.3	This study
Sulfurihydrogenibium str. GV2-1C1[a]	Geyser Valley, Kamchatka, HS	60/6.5	This study
Sulfurihydrogenibium str. UZ1-1C1[a]	Uzon Caldera, Kamchatka, HS	60/6.5	This study
Sulfurihydrogenibium str. UZ1-1C2[a]	Uzon Caldera, Kamchatka, HS	60/6.5	This study
Sulfurihydrogenibium str. UZ3-5[a]	Uzon Caldera, Kamchatka, HS	70/7.4	This study
Aquificaceae			
Aquifex pyrophilus	Marine sediment, Iceland	85/6.8	Huber et al. (1992)
Hydrogenivirga caldilitoris	Coastal hot spring, Japan	75/6.5–7	Nakagawa et al. (2004)
Thermocrinis rubber	Octopus Spring, YNP, HS	80/7–8.5[b]	Huber et al. (1998)
Hydrogenobacter subterraneus	Subsurface water pool, Japan	78/7.5	Takai et al. (2001)
Hydrogenobacter str. 153II6	El Tatio, Chile, HS	60/6.3	This study
Hydrogenobacter str. SS4	Calcite Springs, YNP, HS	70/6.5	This study
Hydrogenobacter str. GV1-4	Geyser Valley, Kamchatka, HS	70/7.4	This study
Hydrogenobacter str. GV2-1C3	Geyser Valley, Kamchatka, HS	70/7.4	This study
Hydrogenobacter str. GV4-1	Geyser Valley, Kamchatka, HS	70/7.4	This study
Hydrogenobaculum acidophilum	Solfatara field, Gunma, Japan	65/3–4	Shima and Suzuki (1993)
Hydrogenobaculum str. Y04AAP1	Artists Paint Pots, YNP, HS	58/4	This study
Hydrogenobaculum str. Y04AAS1	Obsidian Pool, YNP, HS	58/4	This study
Hydrogenobaculum str. Y04ANC1	Nymph Creek, YNP, HS	58/4	This study
Aquificaceae str. CR11	Rincón Volcano, Costa Rica, HS	80/6.7	This study

Temperature and pH indicated correspond to optimal conditions for type species and isolation conditions for strains described in this study
DSV deep-sea vent, *HS* terrestrial hot spring, *YNP* Yellowstone National Park
[a] *aclB* gene sequenced from these strains
[b] pH range for growth, optimum non determined

New sequences were subjected to a basic local alignment search tool (BLAST) search (Altschul et al. 1997) to get an initial identification of similar sequences in the NCBI public database. The ARB program (http://www.mikro.biologie.tu-muenchen.de) was used for 16S rRNA gene sequence alignment and distance-based and parsimony phylogenetic analyses. All 16S rRNA sequences were aligned with the automatic alignment tool of ARB and then manually adjusted according to the secondary structure constraints of the 16S rRNA molecule (Ludwig et al. 1998). Trees were constructed by using the sequences of closely related organisms identified in the ARB database and by BLAST analysis and nearly full-length 16S rRNA sequences of extant Bacteria and Archaea. A filter was generated in order to mask regions with greatest structural variability across taxa, and restrict the dataset to nucleotide sites with higher confidence in homology (~1,300 nt). Overall similarity of sequences was determined with the similarity matrix option available within ARB using the more conserved nucleotides retained after the filter. Phylogenies were constructed of aligned sequences using evolutionary distance (Jukes-Cantor model, with neighbor-joining), parsimony (ARB) and maximum likelihood (fastDNAml and repeated with PAUP) analysis.

ITS and *aclB* gene sequences were aligned with ClustalW and adjusted in MacClade. Other sequences accessible in public databases were included in the alignment (AF252553, AF352545 for ITS and AY553060, AY553054, AB054669 for *aclB* gene). For *aclB*, nucleotide sequences were translated to amino acids prior to analysis and then trimmed to maximize aligned sites across taxa. Insertions and deletions (gaps) were treated as sites of ambiguous resolution. Phylogenetic analyses were performed with PAUP using different algorithms (neighbor-joining, parsimony and ML), except for likelihood (ML) analyses of *aclB*, which were

conducted with PhyML. For all genes, candidate ML models were first compared using the Akaike Information Criterion. The best fitting model for each gene was then used as a starting point for successive approximation of the ML topologies, accounting for invariant sites and modeling rates using a gamma distribution in all cases. As topologies obtained for 16S rRNA, ITS and *aclB* were near identical with different algorithms, only trees based on the distance-based analyses (with neighbor-joining) are presented here (Fig. 1), including bootstrap values as a measure of nodal support.

Accession numbers

Nucleotide sequences have been submitted to GenBank under the accession numbers AM259506 through AM259540, AM259542 through AM259556 and AM260555.

Results and discussion

Numerous new *Aquificales* strains were isolated from deep-sea vents on the CIR (23°S, 69°E), the MAR (23°22′N, 44°57′W), the EPR (9°N, 104W), and from hot springs in Chile, Costa Rica, Kamchatka (Russia) and YNP (USA) (Table 1). The distinctiveness of each new isolate was initially characterized using their 16S rRNA gene sequences. Additionally, we obtained the sequence of the ITS region from these isolates, further expanding the environmental ITS sequences belonging to the *Aquificales* beyond those previously obtained for *Thermocrinis* spp. (Blank et al. 2002). As most described *Aquificales* are chemolithoautotrophs and because the few isolates whose CO_2-fixation pathway has been investigated exploit the rTCA cycle (Beh et al. 1993; Shiba et al. 1985; Deckert et al. 1998; Campbell and Cary 2004; unpublished genome sequences, www.tigr.org), we assumed this pathway is most likely common to all *Aquificales*. One of the central genes in this cycle is the ATP citrate lyase gene. The presence of this gene was screened in our cultures and we were able to successfully amplify the *aclB* gene from many of our *Aquificales* isolates. However, only members of the *Hydrogenothermaceae*, that is, *Persephonella* and *Sulfurihydrogenibium* spp. were included in our *aclB* analysis because our primers (Campbell et al. 2003) failed to amplify *Aquificaceae*-related isolates (Table 1) although there is evidence that they contain this gene (Shiba et al. 1985; Beh

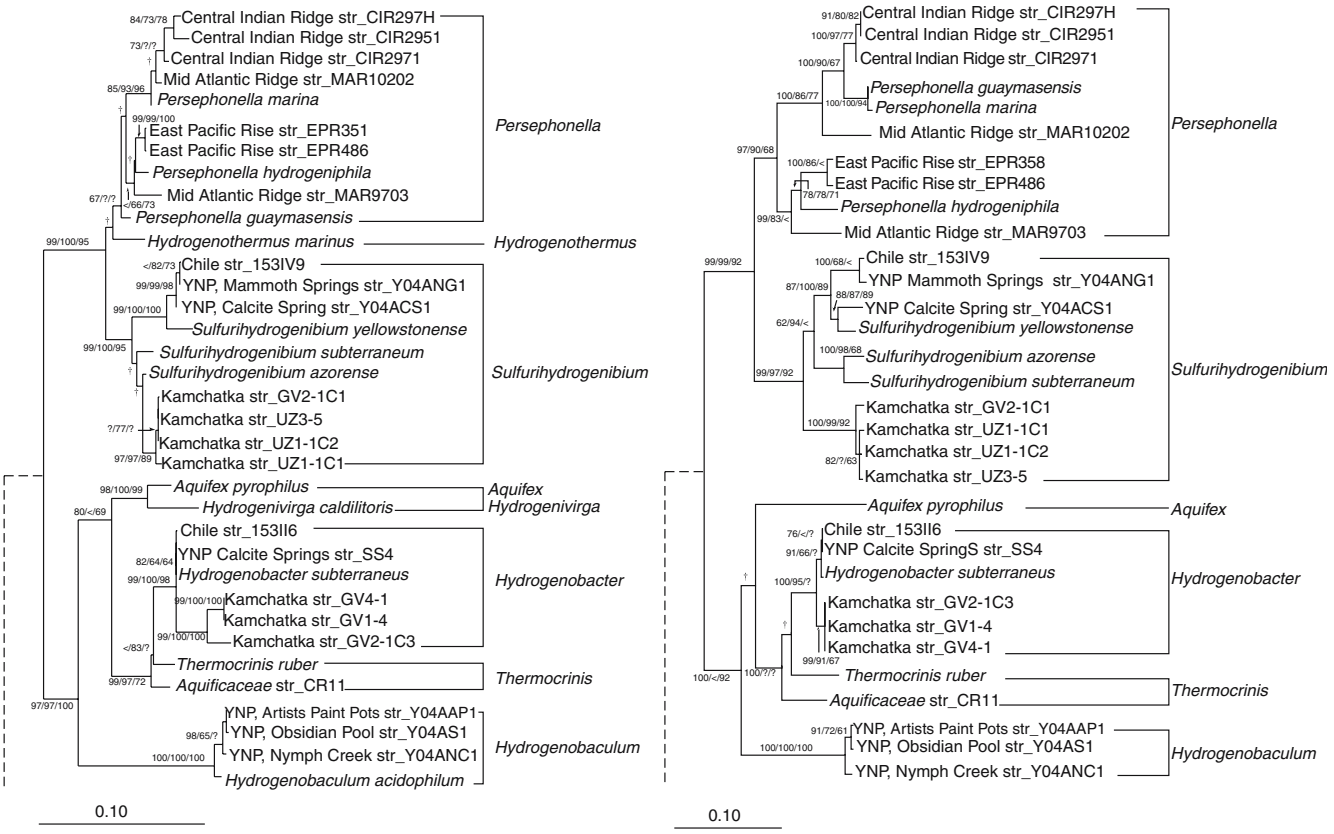

Fig. 1 Neighbor-joining phylogram showing the interrelationships of *Aquificales* isolates inferred from 16S rRNA gene (**a**) and ITS region (**b**). Bootstrap values above 60% are shown in the following order: neighbor-joining (NJ), parsimony (P) and maximum likelihood (ML) phylogenies. Values correspond to 1,000 replicates for NJ and P, or to 100 replicates for ML. The *scale bar* corresponds to 0.1 substitutions per nucleotide position. *Less than symbol* indicates bootstrap below 60%, *question mark* indicates unresolved clade, *dagger* indicates node present but weakly supported by all methods

et al. 1993). Primers amplifying a larger fragment of the *aclB* gene (Takai et al. 2005) do not amplify *Aquificaceae* strains either. Nevertheless, comparisons of the phylogenetic relationships among all strains using these three markers, namely the 16S rRNA gene, the ITS region and the *aclB* gene, were in general consistent, with a few exceptions discussed next.

16S rRNA-inferred phylogeny

The phylogenetic analysis of 16S rRNA gene sequences confirmed two monophyletic lineages corresponding to the two recognized families within this order, the *Aquificaceae* and the *Hydrogenothermaceae* (Eder and Huber 2002) (Fig. 1). Although the *Desulfurobacteriaceae* may be considered a third family (L'Haridon et al. 2006) this lineage was not considered in our analysis. Although some minor differences in the relationships among taxa at the shallowest divergences were observed when using parsimony and ML algorithms, overall the general topology was maintained regardless of which approach was used for phylogeny reconstruction, and in all analyses of 16S rRNA gene, the nodes representing the two main families were supported by high bootstrap values (95 and 99% in the distance-based approach). Furthermore, when 16S rRNA sequences are folded into putative secondary structures based on the model proposed by Noller (1984), large-scale differences in the structural architecture specific for each family, the *Aquificaceae* and the *Hydrogenothermaceae* are evident. The stem loop of Helix 18 (437–497 *Escherichia coli* sequence numbering) within the *Aquificaceae* is similar to *E. coli* and is conserved in structure (length of 62 nt) although not in sequence. This domain is different in members of the family *Hydrogenothermaceae* where a major reduction of multiple nucleotides is found and the terminal is truncated (36 nt). Likewise, this domain reduction is conserved in structure but not in sequence in all members of the *Hydrogenothermaceae* analyzed in this work. Recently, Griffiths and Gupta (2006) have identified molecular signatures in protein sequences for distinguishing the *Aquificales* from other bacteria. Additionally, an *Aquificaceae* signature in the protein synthesis elongation factor EF-Tu was identified. However, the authors compared the DNA sequence of only five strains of *Aquificales* (three members of the *Aquificaceae* and two members of the *Hydrogenothermaceae*), so that the taxonomic scope of this specific signature remains unclear. Moreover, 16S rRNA gene analysis is still the most common tool for phylogenetic characterization of microorganisms; thus, the identified signatures in the 16S rRNA structure provide consistent means for distinguishing species from one family of *Aquificales* to another.

Within the *Hydrogenothermaceae*, two monophyletic branches separate the marine from the terrestrial strains, namely the marine *Persephonella* and *Hydrogenothermus* from the terrestrial *Sulfurihydrogenibium* spp. The three described species of *Persephonella*, *P. marina*, *P. hydrogenophila* and *P. guaymasensis* (Nakagawa et al. 2003; Götz et al. 2002) form a monophyletic group with new isolates from deep-sea vents. Likewise, *Sulfurihydrogenibium* forms a monophyletic branch encompassing *S. azorense*, *S. yellowstonense* and *S. subterraneum*. New terrestrial isolates from YNP, Chile and Kamchatka are placed within this genus. The Yellowstone and Chilean strains are closely related to *S. yellowstonense* (97.6 and 97.8% similarity); however, whether these are strains of the same species cannot be concluded by 16S rRNA similarity alone. Four isolates from two different thermal areas in Kamchatka, Russia, the Geyser Valley and the Uzon Caldera, form a separate lineage within the *Sulfurihydrogenibium* cluster, which on average are 94 and 98% similar to the *S. yellowstonense* and *S. azorense*, respectively.

The *Aquificaceae* includes *Aquifex*, *Hydrogenivirga*, *Hydrogenobacter*, *Thermocrinis*, and the only acidophilic *Aquificales*, *Hydrogenobaculum*. The latter forms the most basal lineage within the *Aquificaceae* although this placement is not strongly supported in all analyses. We isolated three new strains from acidic hot springs in YNP (Artist Paint Pots, Nymph Creek and Obsidian Pool), which clustered with *Hydrogenobaculum*. In addition, five new closely related strains of *Hydrogenobacter* were obtained from Chile, Kamchatka and Calcite Springs (YNP). Both the Chilean and Yellowstone isolates have 16S rRNA gene sequences almost identical to *Hydrogenobacter subterraneus* (99.9 and 100% similarity, respectively), which is a heterotrophic isolate from a subsurface hot water pool in a Japanese geothermal power plant (Takai et al. 2001). Unlike the reports for *H. subterraneus*, these five new strains were able to grow under chemolithoautotrophic conditions (H_2, O_2 and CO_2). We therefore also investigated their ability to grow under aerobic heterotrophic conditions, and indeed the Yellowstone isolate was able to grow on organic substrates (yeast extract, peptone, glucose) and was capable of using the oxidation of thiosulfate as an energy source. Furthermore, under our laboratory conditions, *H. subterraneus* is able to grow autotrophically using hydrogen and oxygen as electron donor and acceptor, respectively (unpublished results). As observed for the *Sulfurihydrogenibium* strains from Kamchatka, the three *Hydrogenobacter* strains from this region clearly formed a separated cluster within the *Hydrogenobacter* lineage.

Another new isolate within the *Aquificaceae* was obtained from a hot spring in the Rincón Volcano (Costa Rica). Based on 16S rRNA comparisons, this new isolate was only 94.5% similar to its closest relative, *Thermocrinis ruber*, and most likely represents a new genus in the family *Aquificaceae*. Furthermore, in all phylogenetic approaches, this new isolate forms a separate branch supported with high bootstrap values in the different analyses, though its separation from *T. ruber* was only strongly supported in parsimony analyses.

ITS- and *aclB*-inferred phylogeny

Numerous studies have shown that phylogenetic analysis of the ITS region provides greater resolution in elucidating phylogenetic relationships among closely related strains and species (reviewed in García-Martínez et al. 1999). Likewise, phylogenies from protein-coding genes often provide additional insights into the relationships within groups and among different lineages sharing similar functional properties, as they can provide information on processes such as lateral gene transfer (Klein et al. 2001). Previously, Blank et al. (2002) found a high diversity in environmental ITS sequences closely related to *T. ruber*, a member of the *Aquificales*. Their results thus suggested that this region of genetic sequence could be highly variable in these microbes. However, we found that the region is conserved in length and sequence across a wider spectrum of different lineages of *Aquificales*, and is likely not a useful marker to differentiate closely related strains as it has been found to be in other groups such as *Firmicutes* (Daffonchio et al. 2003; Cherif et al. 2003), *Alphaproteobacteria* (Kwon et al. 2005) or *Cyanobacteria* (Rocap et al. 2002; Ferris et al. 2003). In the latter case, even differences in length among closely related strains of cyanobacteria were observed. In general, the ITS spacer region can have two, one or no t-RNAs and range in size from around 200 nt to more than 1,200 nt across different microorganisms (García-Martínez et al. 1999; Kwon et al. 2005). We found that within all the *Aquificales* we analyzed the ITS region is conserved in size, being approximately 350 nt in length and includes two t-RNAs (t-RNA-Ile and t-RNA-Ala). Therefore the noncoding spacer regions, which are generally thought to be free from selective constraints and can undergo a higher rate of mutation, are short compared with ITS regions of other groups. The presence of two conserved t-RNAs and reduced spacer regions may explain the relative lack of sequence variability of ITS in the *Aquificales*.

In general, when comparing the topologies generated by 16S rRNA and ITS sequences, the phylogenic estimates of the *Aquificales* relationships appear to be consistent (Fig. 1). Again, the two families *Aquificaceae* and *Hydrogenothermaceae* are reciprocally monophyletic, each generally supported with high bootstrap values. The major clusters of *Persephonella*, *Sulfurihydrogenibium*, *Aquifex*, *Thermocrinis* and *Hydrogenobaculum* show the same topology to results from 16S rRNA gene, and bootstrap values are high for most of the clusters. Yet, subtle changes in the branching patterns were observed for some species, such as *P. guaymasensis* and *Sulfurihydrogenibium* spp. (Fig. 1). The ITS phylogeny also supported the separate branching for the Costa Rican isolate (strain CR11), providing additional evidence that this isolate is very likely a new genus. Like the ITS phylogeny, the *aclB*-based phylogeny was also largely concordant with 16S rRNA, although, in general, *aclB* gene showed less divergence within the *Aquificales* than the 16S rRNA gene and ITS region (data not shown). However, this is not totally surprising as the derived amino acid sequence was used to build the phylogeny with *aclB*. For example, *P. marina* and *P. guaymasensis*, have the same *aclB* gene sequence at the amino acid level. Additionally, the CIR and EPR isolates grouped together in both the 16S rRNA and *aclB* trees but the CIR isolates exhibit a higher diversity in 16S rRNA gene sequence than with *aclB*. The *aclB* analyses provided further support that the new *Sulfurihydrogenibium* strains from Kamchatka are different from *S. azorense*, *S. subterraneum* and *S. yellowstonense*, and thus probably represent new species. Therefore, even though *aclB* results show the limitations of using this gene for differentiating species, *aclB* gene it is still useful as a phylogenetic marker.

The phylogeny of the previously known *Aquificales* and many new strains appears to be consistent when using 16S rRNA gene, ITS region and *aclB* gene, yet the latter two failed to discriminate between closely related strains. Some studies have shown that in some microbial groups, the use of techniques with a higher level of resolution is necessary to differentiate strains. For example, Cho and Tiedje (2000) could not find a strong degree of endemicity in strains of *Pseudomonas* by analyzing 16S rRNA gene and ITS region alone, but they observed a strict endemicity when using repetitive extragenic palindromic PCR genomic fingerprinting with a BOX primer set (BOX-PCR). High-resolution multilocus sequence analysis of eight loci in several strains of *Sulfolobus* (Whitaker et al. 2003) also revealed a high degree of endemicity in this thermophilic archaeon. These approaches have also revealed biogeographical patterns of microbial diversity (Hughes et al. 2006). In our study, the clear phylogenetic clustering of some lineages such as those isolates from Kamchatka suggests that perhaps biogeographical patterns might be revealed for the *Aquificales* if more isolates were analyzed using, for example, multilocus sequencing. Furthermore, with at least four more *Aquificales* genomes already completed (*S. azorense*, *S. yellowstonense* and *P. marina*) or being sequenced (*Hydrogenivirga*; www.moore.org/microgenome), the use of whole-genome microarrays of *Aquificales* will help to determine a more detailed phylogeny of the *Aquificales* and could also be very useful to further investigate the biographical patterns of this group.

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