Isolation of Diverse Members of the Aquificales from Geothermal Springs in Tengchong, China

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Isolation of diverse members of the Aquificales from geothermal springs in Tengchong, China

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INTRODUCTION

The phylum Aquificae is composed of a single order, Aquificales, and three families, Aquificaceae, Hydrogenothermaceae, and Desulfurobacteriaceae (Reysenbach et al., 2005; L’Haridon et al., 2006). Aquificales are present in many terrestrial and marine geothermal systems where they often form multicellular “streamer” assemblages (Huber et al., 1998; Reysenbach et al., 2000, 2005; Takacs et al., 2001; Eder and Huber, 2002; Spear et al., 2005; Hou et al., 2013; Takacs-Vesbach et al., 2013) but can also be prominent members of planktonic microbial communities (Cole et al., 2013; Hou et al., 2013; Murphy et al., 2013). Most members of the Aquificales are obligate or facultative autotrophs (Kawasumi et al., 1984; Stohr et al., 2001; Takai et al., 2001; Eder and Huber, 2002; Aguiar et al., 2004; Caldwell et al., 2010), although at least one isolate was reported to be incapable of autotrophic growth under the conditions that were tested (Takai et al., 2001). Although very few studies have quantified autotrophy in terrestrial geothermal systems inhabited by Aquificales (Boyd et al., 2009), Aquificales are broadly hypothesized to be important primary producers and are capable of using a variety of inorganic compounds to fuel chemolithotrophy, including diverse electron donors (H2, S2−, SO42−, Fe3+, AsO43−) and terminal electron acceptors (O2, NO3−, SO42−, Fe3+, AsO43−, SO32−; Stohr et al., 2001; Takai et al., 2001; Eder and Huber, 2002; O’Neill et al., 2008).

Two families of Aquificales dominate in terrestrial geothermal systems, the Aquificaceae and Hydrogenothermaceae. The Aquificaceae includes three genera that are abundant in terrestrial systems: Hydrogenobacter, Thermocrinis, and Hydrogenobaculum (Reysenbach, 2001; Takacs-Vesbach et al., 2013). Hydrogenobacter and Thermocrinis are closely related and are capable of axenic growth at circumneutral pH to ≥85°C (Kawasumi et al., 1984; Takai et al., 2001; Eder and Huber, 2002) and ≥89°C (Huber et al., 1998; Eder and Huber, 2002; Caldwell et al., 2010), respectively. In contrast, known isolates of Hydrogenobaculum are acidophilic (optimum pH 3–4) and have lower growth temperature ranges, with optima between 60 and 70°C (Shima and Suzuki, 1993; D’Imperio et al., 2008). The family Hydrogenothermaceae includes a single...
genus that is prominent in many terrestrial geothermal systems, *Sulfurihydrogenibium*, with known isolates capable of growth to ≥75°C at circumneutral pH (5.0–8.8; O’Neill et al., 2008).

Yunnan Province, in southwest China, has a large number of geothermal springs, particularly in Tengchong County, which is located within the Indo-Burma Range along the central-western border between Yunnan Province and Myanmar. Geothermal activity in Yunnan Province is typically located along arched fault structures and circular depressions and is likely fueled by latent heat from tectonic activity associated with the subduction of Tethys Ocean lithosphere (Liao and Guo, 1986; Wang et al., 2008). The largest and best-known geothermal area in Tengchong is the Rehai (“Hot Sea”) Geothermal Field, with springs reaching the boiling point (~95°C at ~1,500 m elevation) and spanning a pH range of 2.5–9.4 at high temperature (>80°C; Figure 1; Table 1; Hedlund et al., 2012). A large number of Bacteria and Archaea have been isolated from Rehai springs, particularly thermophilic members of the Firmicutes (Bacillales, Thermoaerobiales, Clostridiales), Deinococcus-Thermus phylum (Thermus), and Crenarchaeota (Sulfolobales) (reviewed in Hedlund et al., 2012). However, despite recent cultivation-independent studies suggesting that *Aquificales* are abundant in nearly all high-temperature sites in Rehai (Pagaling et al., 2012; Hou et al., 2013; Song et al., 2013; Briggs et al., 2014), there are no published reports of the isolation or characterization of *Aquificales* from Rehai or anywhere in China.

In this study, we isolated *Aquificales* from sites in Tengchong known to host abundant *Aquificales* populations and sites with abundant streamer growth that were deemed likely to host *Aquificales*. The strains belong to the genera *Hydrogenobacter*, *Hydrogenobaculum*, and *Sulfurihydrogenibium*, and possibly a new genus within the *Hydrogenothermaceae*. Although most of the
strains likely represent new taxa, their general physiological traits are similar to known members of these genera, including variable capacity for aerobic hydrogen oxidation via the "knallgas reaction," chemolithotrophic oxidation of sulfur compounds, and anaerobic respiration of nitrate.

**MATERIALS AND METHODS**

**SAMPLE COLLECTION, ENRICHMENT, AND ISOLATION**

Sediment, streamer, and mat samples were collected from five hot springs located in the Rehai Geothermal Field in Tengchong County, Yunnan Province, China (Figure 1). Prior to sampling, the temperature and pH were measured at the precise sampling location with a field-calibrated pH probe with temperature correction (LaMotte five Series, Chestertown, MD, USA). Detailed water chemistry and microbial community composition at most of the sampling locations on several previous sampling trips has been reported elsewhere (Hou et al., 2013; Briggs et al., 2014).

Samples from which strains T-2, T-5, T-7, and T-8 were isolated were collected aseptically and transferred into 25 mL Balch tubes containing 5 mL modified MSH medium (Caldwell et al., 2010) containing S₀ and S₂O₃²⁻ and adjusted to pH 8.0 (T-2), 6.5 (T-5 and T-7), or 7.5 (T-8). Tube headspace was either H₂:CO₂ (80:20) for strains T-2, T-7, and T-8, or N₂:CO₂ (80:20) for strain T-5, supplemented with 4% v/v O₂. The tubes were stored and transported at room temperature. Once in the lab, the Balch tubes were incubated at 80°C (T-2) or 70°C (T-5, T-7, and T-8) and passaged in the same medium under the same conditions. To obtain pure cultures of strains T-2, T-7, and T-8, positive enrichments were streaked for isolation onto plates containing GBS salts medium (Dodsworth et al., 2014) containing thiosulfate (1 mM added as Na₂S₂O₃·5H₂O) and solidified with Gelrite (0.8% mass/vol, supplemented with 4 g/L MgCl₂·6H₂O (Serva, Heidelberg)] and incubated at 70°C in modified two quart Bandit pressure pots (C.A. Technologies). Pressure pot headspace consisted of ~2 L anaerobic chamber gas (N₂:CO₂:H₂ at 90:5:5) supplemented with 200 mL H₂:CO₂ (80:20) and 100 mL air. Isolated colonies were re-streaked two times to ensure purity. Strain T-5 was isolated using an extinction-to-dilution method that was repeated at least seven times. For all strains, purity was confirmed through microscopic observation and sequencing of the 16S rRNA gene, following the general approach described previously (Nakagawa et al., 2005).

The sample from which strain T-6 was isolated was aseptically transferred in the field into a 25 mL Balch tube containing 10 mL of DSMZ medium 743 (modified by replacing S₀ with 30 μM Na₂S, pH 3), given a headspace of N₂:CO₂:H₂/air (30:40:20:10), and incubated in the spring. Following growth, the tube was transported to the lab without temperature control. For isolation, 1 mL of the enrichment culture was inoculated into 10 mL of the same medium with the same headspace as in initial enrichment. A pure isolate was obtained by three rounds of dilution to extinction and verified through microscopic observation and sequencing of the 16S rRNA gene.

**GROWTH CHARACTERISTICS**

The capacity for growth of the strains on electron donors and electron acceptors commonly used by *Aquificales* was determined by growing each strain under conditions that permitted good growth, as determined by phase-contrast microscopy. In all cases, growth was determined by direct cell counts using a Petroff-Hauser counting chamber and a phase-contrast microscope. All experiments were performed in triplicate along with positive and negative controls. Strains T-2, T-7, and T-8 were routinely grown at 70°C in 5 mL volumes of GBS salts medium (Dodsworth et al., 2014) with an N₂:H₂:CO₂/air (75:17:4:1) headspace or in 25 mL Balch tubes without shaking. The medium was adjusted to pH 8.0, 7.2, and 6.6 for T-2, T-7, and T-8, respectively. Strain T-5 was routinely grown in a modified MSH medium (Caldwell...
Table 2 | 16S rRNA gene identity to closest cultivated relatives.

| Organism                          | Closest cultivated relative          | % Identity | Accession numbers   |
|-----------------------------------|--------------------------------------|------------|---------------------|
| Hydrogenobacter sp. T-2           | “Hydrogenobacter subterraneus” HGP1T | 96.6       | NR_024729.1         |
| Hydrogenobacter sp. T-8           | “H. subterraneus” HGP1T              | 97.2       | NR_024729.1         |
| Hydrogenobaculum sp. T-6          | Hydrogenobaculum sp. Y04AA81         | 95.3       | CP001130.1          |
| Hydrogenothermaceae strain T-5    | S. rodmanii UZ3-5T                   | 94.6       | NR_042515.1         |
| S. subterraneum T-7               | S. subterraneum HGMK-1T              | 99.4       | NR_036883.1         |

Phylogenetic analysis based on near-complete 16S rRNA genes showed that the strains belonged to the families *Aquificaceae* and *Hydrogenothemaceae*. Two *Hydrogenobacter* strains were isolated, designated T-2 and T-8, from sites differing in pH by > 2.5 units. They were grown in media with pH similar to their environmental source, although both were closely related to *Hydrogenobacter subterraneus* (Table 2; Figure 2). Both strains belonged to a species-level (98.65% identity; Kim et al., 2014) operational taxonomic unit (OTU) that comprised >50% of 16S rRNA gene sequence tags in either sediments or the bulk water in most circumneutral geothermal sites in both Rehai and Ruidian (Dientan) geothermal fields (Hou et al., 2013), including streamer
and sediment communities in Guminquan, from which strain T-2 was isolated. However, the most abundant sequence within that OTU shared only 98.84% identity with strains T-2 and T-8, whereas the identical sequence to T-2 and T-8 was a rare variant in the cultivation-independent datasets. The other isolate belonging to the \textit{Aquificaceae}, strain T-6, was isolated from the acidic pool, Diretiyanqu. Strain T-6 branched within the genus \textit{Hydrogenobaculum} but was distant from the only validly described species, \textit{Hydrogenobaculum acidophilus}, as well as other isolates from Yellowstone National Park (Table 2; Figure 2). Strain T-6 belonged to an OTU that comprised 31 to 66% of 16S rRNA gene sequence tags from pools ranging from 55 to 65°C from Diretiyanqu, the system of small acidic pools from which the strain was isolated (Hou et al., 2013). Within these systems, the dominant OTU was identical to T-6.

Sulfurihydrogenibium strain T-7 was closely related to \textit{Sulfurihydrogenibium subterraneum} HGMK-1T, and strain T-7 branched with 16S rRNA gene clones from Asia (Japan and Taiwan), potentially representing a species exclusive to Asia (Figure 2). T-5, the other strain that branched from within the \textit{Hydrogenothermaceae}, was only distantly related to cultivated strains of \textit{Sulfurihydrogenibium} and \textit{Venetivibrio} and branched with 16S rRNA gene clones from hot springs in China and Thailand (Table 2; Figure 2; JX298759 and KC831413, unpublished). Aside from strain T-7, the 16S rRNA gene identity between each new isolate and the most closely related species was well below the 16S rRNA gene identity threshold suggested to delimit bacterial species (98.65%; Kim et al., 2014), and strains T-6 and T-5 were also below the median 16S rRNA gene identity circumscribing bacterial genera (Yarza et al., 2014). Formal taxonomic treatment of these isolates will be determined pending detailed physiological and genomic analysis.

With the exception of T-5, all strains were capable of chemotrophic growth with H\textsubscript{2} as the electron donor under microaerophilic conditions (Table 3). Both \textit{Hydrogenobacter} strains also used S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-} as an electron donor and \textit{Hydrogenobacter} sp. T-2 additionally used S\textsuperscript{0} and acetate as electron donors. \textit{Hydrogenobacter} sp. T-8 grew anaerobically by reducing nitrate. Neither nitrous oxide nor dinitrogen were identified as products of nitrate reduction. \textit{Hydrogenobacter} sp. T-8 grew anaerobically by reducing nitrate. Neither nitrous oxide nor dinitrogen were identified as products of nitrate reduction. \textit{Hydrogenothermaceae} strain T-6 was capable of microaerophilic growth with S\textsuperscript{2} and S\textsuperscript{2}O\textsubscript{3}\textsuperscript{2-} as alternative electron donors. T-5 could only use sulfur or thiosulfate as electron donors and O\textsubscript{2} as the electron donor.
**Table 3 | Media for routine growth and growth characteristics for Aquificales strains from Tengchong hot springs.**

| Organism                  | Medium for routine growth (gas phase vol.) | Temperature (°C) | pH | Electron donors* | Electron acceptors |
|---------------------------|-------------------------------------------|------------------|----|------------------|-------------------|
| *Hydrogenobacter* sp. T-2 | GBS salts medium (N₂/H₂/CO₂/air; 75:17:4:4) | 70               | 8.0| H₂, S₂O₃²⁻, S⁰ | acetate, O₂       |
| *Hydrogenobacter* sp. T-8 | GBS salts medium (N₂/H₂/CO₂/air; 75:17:4:4) | 70               | 6.6| H₂, S₂O₃²⁻       | O₂, NO₃⁻         |
| *Hydrogenobaculum* sp. T-6 | DSMZ 743 medium (N₂/CO₂/H₂/air; 30:40:20:10) | 60               | 3.0| H₂, S³⁻, S⁰     | O₂               |
| *Hydrogenothermaceae* strain T-5 | Modified MSH medium (CO₂/O₂; 76:4) | 70               | 6.5| S₂O₃²⁻, S⁰      | O₂               |
| *S. subterraneum* T-7   | GBS salts medium (N₂/H₂/CO₂/air; 75:17:4:4) | 70               | 72 | H₂, S₂O₃²⁻, S⁰ | O₂               |

*Electron donors and acceptors that yielded positive growth, defined as a mean cell count of >5.0 × 10⁶ cells/mL for triplicate growth experiments. All growth experiments were conducted in tandem with triplicate positive and negative controls.

**DISCUSSION**

*Aquificales* are globally distributed and often abundant in both marine and terrestrial geothermal systems where they likely play important roles in C, N, H, and S cycles. Recent cultivation-independent censuses of Bacteria and Archaea in hot springs in Tengchong County, China suggested the wide distribution of *Aquificales* in the region, particularly in the Rehai Geothermal System, where *Aquificales* dominated many 16S rRNA gene pyrotag datasets generated using a few different primer sets and on several different sampling campaigns (Pagaling et al., 2012; Hou et al., 2013; Song et al., 2013). Both 16S rRNA gene pyrotag data and phylochip data suggest that *Hydrogenobacter* is a dominant member of most circumneutral to alkaline springs in Rehai (pH 8.1–9.4; Hou et al., 2013; Song et al., 2013; Briggs et al., 2014), including large growths of white streamer material in springs Gumingquan and Jiemeiquan ([Figure 1A; Hou et al., 2013; Briggs et al., 2014]). Strains T-2 and T-8 shared 98.84% 16S rRNA gene sequence identity across the V4 region, suggesting they may belong to the same species as the dominant *Hydrogenobacter* OTU in the springs. However, extrapolation of physiological traits of these strains to the abundant natural populations must be done with caution, since even T-2 and T-8 were different with regard to both electron donor and acceptor use, despite being nearly identical across the near-complete 16S rRNA gene. High intraspecies variation in respiratory capacity may be a common feature in the *Aquificales* (D’Império et al., 2008). The electron donors and acceptors used by the *Hydrogenobacter* isolates are similar to those described for other members of the genus (Kawasumi et al., 1984; Takai et al., 2001; Eder and Huber, 2002). “*H. subterraneus*,” the most closely related isolate described in detail, is similar in its ability to use reduced sulfur compounds as electron donors; however, “*H. subterraneus*” is unable to use H₂ as an electron donor and appears to be incapable of autotrophic growth (Takai et al., 2001). The genus *Thermocrinis*, which often forms conspicuous streamer growth (Reysenbach et al., 1994; Huber et al., 1998; Eder and Huber, 2002), has only been detected in one 16S rRNA gene census at Rehai (Song et al., 2013) and was not detected by other 16S rRNA gene PCR censuses and phylochip analysis (Pagaling et al., 2012; Hou et al., 2013; Briggs et al., 2014). The sporadic detection of *Thermocrinis* in Rehai may explain why cultivation experiments described here did not yield *Thermocrinis* cultures.

Cultivation-independent surveys in Tengchong also identified abundant *Hydrogenobaculum* populations in Rehai springs with pH < 4, particularly within silica sand-dominated acidic pools in Diretiyanqu and Zhenzuquan (Hou et al., 2013; Song et al., 2013; Briggs et al., 2014). *Hydrogenobaculum* strain T-6, used both H₂ and reduced S compounds as electron donors (S²⁻, S⁰, and S₂O₃²⁻). These compounds are widely used by *Hydrogenobaculum* isolates from different locations (Shima and Suzuki, 1993; Donahoe-ChristianSENs et al., 2004; D’Império et al., 2008), although isolates from Yellowstone National Park are heterogeneous with regard to their ability to oxidize H₂ (D’Império et al., 2008). Arsenite oxidation and the encoding structural genes, aioBA, have been documented for some *Hydrogenobaculum* isolates (Donahoe-ChristianSENs et al., 2004; Clingenpeel et al., 2009; Romano et al., 2013). The *aioBA* genes have also been cloned from natural geothermal environments, including those inhabited by *Hydrogenobaculum* (Clingenpeel et al., 2009; Hamamura et al., 2009) that are similar to those in...
our study site with regard to temperature and pH. However, strain T-6 was unable to oxidize arsenite under the conditions tested and so perhaps it is similar in regard to Yellowstone strain Y04AAS1, which lacks recognizable aioBA (Romano et al., 2013) and has not been reported to oxidize arsenite. Lack of arsenite oxidation capability may reflect the relatively low concentrations of total arsenic at this site [50–200 ppb (Hou et al., 2013)].

In contrast to the Aquificaeae, cultivation-independent surveys have suggested a low abundance of Hydrogenothermaceae, including sequences that were related to Hydrogenothermus, Persephonella, and Sulfurihydrogenibium (Pagaling et al., 2012; Song et al., 2013; Briggs et al., 2014). However, two springs not included in published cultivation-independent studies of Rehai yielded sequences that were related to S. subterraneum. Strain T-7 was very closely related to S. subterraneum HGMK-1T. The electron donors used by strain T-7 were identical to those used by S. subterraneum HGMK-1T (Nakagawa et al., 2005), although T-7 appeared to be more restricted in its use of terminal electron acceptors. Strain T-5 could not grow on any complex organics, and could only use sulfur or thiosulfate as electron donors in the presence of oxygen as an electron acceptor.

CONCLUSION

This study expands both the geographic and phylogenetic coverage of Aquificales cultivated from terrestrial geothermal springs. This study is particularly important within the context of the study of thermophilic microbial communities in Tengchong County because abundant evidence from cultivation-independent studies implicate the Aquificales as widely distributed and abundant microorganisms with potential roles in several biogeochemical cycles. Known phenotypic variability within the Aquificales notwithstanding, these studies provide a strong foundation for understanding the potential roles of these organisms in C, N, S, and H cycles in the Rehai Geothermal System. The Aquificales isolates described here likely represent novel species of Hydrogenobacter (strains T-2 and T-8) and Hydrogenobaculum (strain T-6) and a new genus in the Hydrogenothermaceae (strain T-5). Further work is underway to thoroughly taxonomically describe these novel organisms.

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