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ORIGINAL PAPER

A molecular and physiological survey of a diverse collection of hydrothermal vent *Thermococcus* and *Pyrococcus* isolates

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Abstract Strains of hyperthermophilic anaerobic hydrothermal vent archaea maintained in the culture collection assembled by Holger Jannasch at the Woods Hole Oceanographic Institution between 1984 and 1998 were identified and partially characterized by Denaturing Gradient Gel Electrophoresis, 16S rRNA gene sequencing, and by growth tests at different temperatures and on different organic carbon and nitrogen sources. All strains were members of the genera *Thermococcus* and *Pyrococcus.* The greatest phylogenetic diversity was found in strains from a single Guaymas Basin core isolated by serial dilution from four different depth horizons of heated sediment incubated at the corresponding in situ temperatures. In contrast, geographically distinct vent locations and sample materials yielded a lower diversity of isolates when enriched under uniform temperature regimes and without prior dilution of the source material.

Keywords Deep sea hyperthermophiles · Taxonomy · Isolation and characterization · *Thermococcus* · *Pyrococcus*

Introduction

The genera *Thermococcus* and *Pyrococcus* are the most frequently isolated hyperthermophilic archaea; at least 25 recognized *Thermococcus* spp. have been isolated, and continue to be isolated, from deep and shallow marine hydrothermal vents (Zillig and Reysenbach 2001; Kuwabara et al. 2007). Members of the Thermococcales assimilate and ferment complex organic compounds, such as yeast extract, tryptone, peptone, casein, diverse sugars and peptides. Fermentative growth is enhanced by addition of elemental sulfur, or in the case of some species, requires sulfur; sulfur acts as an auxiliary electron acceptor, and extracellular hydrogen sulfide accumulates in high concentrations in growth media. In addition to heterotrophy, some species and strains have the potential for

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carboxydotrophic growth (Miroshnichenko and Bonch-Osmolovskaya 2006; Lee et al. 2008). Members of the genera *Thermococcus* and *Pyrococcus* grow quickly and robustly on a wide range of liquid anaerobic laboratory media supplemented with sulfur, and are isolated frequently from all kinds of hydrothermal vent sample materials, including the subsurface underneath hydrothermal vents, that they are viewed as indicator species of hydrothermal activity (Kelley et al. 2002).

A collection of *Thermococcus* and *Pyrococcus* isolates from different hydrothermal vent sites in the Pacific and Atlantic oceans was established in the Jannasch laboratory at the Woods Hole Oceanographic Institution (WHOI), between 1984 and 1998. Some of these strains are well documented in the literature and have been used for extensive physiological studies of growth and survival under hydrothermal vent conditions, especially *Pyrococcus* strain GBD from Guaymas Basin (Jannasch et al. 1988, 1992; Edgcomb et al. 2004, 2007; Lloyd et al. 2005). However, most of these strains have remained unstudied and unpublished. The goal of this study is to provide a partial overview of this strain collection, which remains at WHOI. The isolates were characterized based on 16S rRNA gene sequencing, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments, growth temperature and range of utilizable organic substrates. We examined isolates from the culture collection for physiologically and phylogenetically related groups, and we evaluated how different isolation strategies influenced the diversity of the strains that were obtained.

Materials and methods

Strain isolation and preservation

Most strains were enriched and isolated on half-strength no. 2216 Marine Broth (Difco; referred to as 2216 medium) with additions of 1% (wt/vol) elemental sulfur that was heat-sterilized by repeated steaming at 100°C (Jannasch et al. 1988). The 2216 Marine Broth was diluted to half-strength with Turk's Island (TI) mineral medium (Jannasch et al. 1992). TI medium is an artificial seawater (ASW) consisting of a salt mixture of 28.15 g of NaCl, 5.5 g of $MgCl_2 \times 6H_2O$, 6.9 g of $MgSO_4 \times 7H_2O$, 0.7 g of KCl, mixed in 950 ml water; and 1.45 g of $CaCl_2 \times 2H_2O$, dissolved separately in 50 ml water and combined to one liter. After purging with oxygen-free nitrogen, one liter of Turk's Island ASW was combined with the same volume of 2216 Marine Broth (37.4 g/l DW); the pH was adjusted with 9.63 g/l (20 mM) PIPES buffer and titrated to 7.5 with 4 N HCl. As redox indicator, 0.4 ml of 0.2% resazurin solution was added. The medium was autoclaved, filtered through a 0.2-μm glass filter to remove solids, and either stored anaerobically until use or dispensed anaerobically into 15 ml Hungate vials, each of which contains a pea-sized amount of steam-sterilized elemental sulfur (Jannasch et al. 1988). In some enrichments (Table 1), T-5 medium was used, a modified TYEG medium (Jannasch et al. 1992). T-5 medium consists of a seven salt artificial seawater base (20 g NaCl, 3 g of $MgCl_2 \times 6H_2O$, 6 g of $MgSO_4 \times 7H_2O$, 1 g of $(NH_4)_2SO_4^{2-}$, 0.2 g of $NaHCO_3$, 0.3 g of $CaCl_2 \times 2H_2O$, 0.5 g of KCl and 0.42 g/l KH_2PO_4), 3 g/l each of Trypticase, glucose and yeast extract, 5 ml of trace element solution per liter (Wolin et al. 1963) and 5.0 ml of vitamin mix per liter. The vitamin mix contained 10 mg/l each niacin, calcium pantothenate, *p*-aminobenzoic acid, thiamine, riboflavin, pyridoxine, cobalamin, alpha-lipoic acid; and 4 mg/l each folic acid and biotin. The solution was filter sterilized and stored in the dark at 4°C (Bazylinski et al. 1989). The medium was filter sterilized, reduced by addition of 3.2 mM sodium sulfide and set at pH 7.2. Active *Thermococcus* and *Pyrococcus* enrichments and pure cultures grown in sulfur-containing media contained enough biologically produced sulfide to reduce fresh tubes of medium without prior Na_2S addition.

Sampling and isolation of Guaymas Basin strains

Sediments from hydrothermally-active Guaymas Basin were collected by push core on Alvin Dive 3203 on April 26, 1998 (Core No. 2) taken near the Everest Mound area in the Southern vent field of Guaymas Basin (RV Atlantis/Alvin, April 25–May 5, 1998). The in situ temperature gradient was determined in 5-cm intervals, using the external Alvin temperature probe. The push core was returned to the surface and sliced into 1-cm layers. For cultivations of *Thermococcus* and *Pyrococcus* spp., 1 ml of sediments were set aside from the four sediment layers at 5–6, 9–10, 13–14 and 19–20-cm depth that corresponded to temperatures near 75, 85, 95 and 105°C. Within 24 h of sediment core recovery, four dilution series were inoculated in Hungate tubes containing sulfur-amended half-strength 2216 medium, and these were incubated shipboard in water baths at the corresponding in situ temperatures, with the exception of the 105°C sample which was incubated at 100°C. Growth was detected by turbidity and sulfide production within 24 h. These shipboard dilution series were performed with 2216 half-strength medium, made by mixing 2216 full-strength medium mixed with equal volumes of GB (Guaymas Basin) basal medium, consisting of 25 g of NaCl, 6.8 g of $MgSO_4 \times 7H_2O$, 0.3 g of $CaCl_2 \times 2H_2O$, 0.5 g of KCl, 0.09 g KBr and 1.0 g NH_4Cl per liter of distilled water. The basal GB medium was mixed with 2216 solution, the pH was adjusted with

Table 1 *Pyrococcus* and *Thermococcus* isolates of the former Jannasch strain collection, with source material, geographic origin and year of isolation, and identification into DGGE Groups 1–8

Isolate	DGGE group	Genus	Medium and temperature (°C) for enrichment		Inoculum source	Place and year	Growth at		
							90°C	96°C	99°C
A. Characteristics of *Thermococcus* and *Pyrococcus* strains in DGGE Groups 1 and 2									
GB49	1	Tc	TI-2216/2	80[a]	Surficial 0–7.5 cm sediment	Guaymas 1985	+	−	−
GB121	1	Tc	TI-2216/2	80[a]	Black smoker sulfide	Guaymas 1985	+	−	−
MA2.27	1	Tc	TI-2216/2	90	Chimney sulfide, thin wall	MAR Tag 1993	+	−	−
MA2.28	1	Tc	TI-2216/2	90	Inner scrapings fr. chimney w. shrimp	MAR Tag 1993	+	−	−
MA2.29	1	Tc	TI-2216/2	90	Chimney sulfide subsurface scraping	MAR Tag 1993	+	+	−
MA2.33	1	Tc	TI-2216/2	90	Chimney sulfide scraping, hard surface	MAR Tag 1993	+	−	−
9°N2.21	1	Tc	TI-2216/2	90	Slurp sample from chimney surface	9°N EPR 1994	+	+	−
9°N2.22	1	Tc	TI-2216/2	90	Chimney sulfide subsurface scraping	9°N EPR 1994	+	−	−
9°N2.23	1	Tc	TI-2216/2	90	Slurp sample chimney, subsurface	9°N EPR 1994	+	−	−
9°N2.24	1	Tc	TI-2216/2	90	*Alvinella* worm tube	9°N EPR 1994	+	−	−
9°N2.25	1	Tc	TI-2216/2	90	Chimney sulfide subsurface scraping	9°N EPR 1994	+	−	−
9°N2.26	1	Tc	TI-2216/2	90	*Alvinella* worm tube	9°N EPR 1994	+	−	−
GB1	1	Tc	GB-2216/2	90	*Alvinella* worm tube	21°N EPR 1998	+	−	−
GB2	1	Tc	GB-2216/2	90	Black smoker rock	21°N EPR 1998	+	−	−
GB3	1	Tc	GB-2216/2	90	Smoker worm tube	21°N EPR 1998	+	−	−
GB16	1	Tc	GB-2216/2	90	"Artichoke" smoker	21°N EPR 1998	+	−	−
GB17	1	Tc	GB-2216/2	90	0 to 7.5 cm sediment	Guaymas 1998	+	−	−
GB19	1	Tc	T5	90	15 to 22 cm sediment	Guaymas 1998	+	−	−
GB20	1	Tc	GB-2216/2	90	Black Smoker surface w. sulfur mat	Guaymas 1998	+	−	−
GB22	1	Tc	AG-YE	90	15 to 22 cm sediment	Guaymas 1998	+	−	−
GBA	2	Pc	TI-2216/2	99	Black Smoker subsurface scrapings	Guaymas 1988	+	+	+
GBC	2	Pc	TI-2216/2	99	Black Smoker subsurface scrapings	Guaymas 1988	+	+	+
GBD	2	Pc	TI-2216/2	99	Black Smoker subsurface scrapings	Guaymas 1988	+	+	+
G1C	2	Pc	TI-2216/2	96	Friable Black Smoker subsurface	Guaymas 1991	+	+	+
G1G	2	Pc	TI-2216/2	96	Flange smoker rock	Guaymas 1991	+	+	+
G1H	2	Pc	TI-2216/2	99	Sediment below *Beggiatoa* mat	Guaymas 1991	+	+	+
G1 J	2	Pc	TI-2216/2	99	Chimney with *Alvinella* tubes	Guaymas 1991		+	+
G1 K	2	Pc	TI-2216/2	96	*Alvinella* worm tube casing	Guaymas 1991	+	+	+

Table 1 continued

Isolate	DGGE group	Genus	Medium and temperature (°C) for enrichment		Inoculum source	Place and year	Growth at		
							90°C	96°C	99°C
G1L	2	Pc	TI-2216/2	96	Black Smoker chimney rock	Guaymas 1991	+	+	+
MA2.31	2	Pc	TI-2216/2	100	Hot smoker sulfide subsurface scraping	MAR Snake Pit 1993	+	+	−
MA2.32	2	Pc	TI-2216/2	100	Hot smoker sulfide subsurface scraping	MAR Snake Pit 1993	+	−	−
MA2.34	2	Pc	TI-2216/2	100	Black smoker sulfide	MAR Snake Pit 1993	+	+	−
GB8.25	2	Pc	GB-2216/2	99	Black Smoker surface	21°N EPR 1998	+	+	+
GB8.27	2	Pc	GB-2216/2	99	7.5 cm sediment	Guaymas 1998	+	+	−
C2	2	Pc	GB-2216/2	95	Sediment core at 14 cm depth (10^2 ml^{-1})	Guaymas 1998	+	−	−
B. Characteristics of *Thermococcus* and *Pyrococcus* strains in DGGE Groups 3 to 8									
GB18	3	Tc	GB-2216	90	7.5 cm Sediment	Guaymas 1998			
GB24	3	Tc	T5	90	7.5 cm Sediment; same core as GB-18	Guaymas 1998			
GB50	4	Tc	TI-2216/2	80[a]	0–7.5 cm Sediment	Guaymas 1985			
11°NA5	5	Tc	TI-2216/2	77	Worm tube casing homogenate	11°N EPR 1984			
11°NVb	5	Tc	TI-2216/2	77	Worm tube casing homogenate	11°N EPR 1984			
GB.128	6	Tc	TI-2216/2	80	Black Smoker surface scrapings	Guaymas 1985			
B4	6	Tc	GB-2216/2	85	Sediment core at 10-cm depth (10^4 ml^{-1})	Guaymas 1998			
A4	7	Tc	GB-2216/2	75	Sediment core at 5-cm depth (10^4 ml^{-1})	Guaymas 1998			
9°N2.20	7	Tc	TI-2216/2	90	Chimney subsurface sulfide scraping	9°N EPR 1994			
A2	8	Tc	GB-2216/2	75	Sediment core at 5-cm depth (10^2 ml^{-1})	Guaymas 1998			
B1	8	Tc	GB-2216/2	85	Sediment core at 10-cm depth (10^1 ml^{-1})	Guaymas 1998			

Tc Thermococcus, *Pc* Pyrococcus

[a] Enriched at 80°C and subsequently grown at 90°C

HCl, resazurin was added, and the medium was autoclaved and filtered as described for half-strength marine broth. The medium was then reduced with 10% (w/vol) Na_2S stock solution to a final concentration of 400 μM, and the pH was readjusted to 7.5. Other Guaymas 1998 strains were enriched on different media, generally within 24 h of sampling, but without serial dilution (Table 1).

Regrowth of stock cultures

All *Pyrococcus* and *Thermococcus* stock cultures were preserved in reduced half-strength 2216 medium with 10% DMSO under liquid nitrogen. Stocks were regrown by injecting 0.5 ml of medium from a gently shaken stock tube into a N_2-flushed Hungate tube with 5 ml medium and 1 g steam-sterilized elemental sulfur. The medium was reduced by adding Na_2S to a final concentration of ca. 300 μM (50–70 μl of a 1% $Na_2S \times 9H_2O$ solution per 10 ml volume). Two stock tubes, grown from the original isolate at different times, were used to regrow each isolate. The resulting cultures were incubated for 24 h in a 90°C water bath, and monitored for growth by observing turbidity and by using oil immersion microscopy. After 12–24 h incubation, the regrown strains were removed from the water bath and stored at 4°C.

DNA extraction, PCR and DGGE

DNA was isolated by hot phenol extraction and ethanol/NaCl precipitation, followed by PCR and DGGE of 16S rRNA genes as described previously (Teske et al. 2000), but with the modification of using archaea-specific primers for PCR amplification of a 550 bp DGGE fragment of the 16S rRNA gene (forward primer ARC344, 5′-ACGGG GYGCASCAGGCGCGA-3′ at *E. coli* 16S rRNA position 344–363, and reverse primer ARC915, 5′-GTGCY CCCCCGCCAATTCC T-3′ at *E. coli* 16S rRNA position 915–934). These DGGE fragments were run for 12 h at 100 V on a 30–70% denaturant gel.

For PCR amplification and sequencing of the near-complete 16S rRNA gene, primers Arc-8F (5′-TCC GGTTGATCCTGCC-3′) and Arc-1492R (5′-GGCTAC CTTGTTACGACTT-3′) were used. Each PCR sample consisted of 5 μl of 10× PCR buffer, 5 μl 2 mM dNTP, 5 μl 10 mM primer ARC-8f, 5 μl 10 mM primer ARC-1492R, 1 μl of 1U/μl *Taq* polymerase, 27 μl sterile ddH_2O, and 2 μl of the purified chromosomal DNA. PCR conditions in a Perkin-Elmer thermocycler were set to 3 min denaturation at 94°C, followed by 30 cycles of amplification (20 s at 94°C, 1 min of annealing at 52.5°C and 2 min of elongation at 72°C) and 10 min of terminal elongation at 72°C, followed by cooling at 4°C.

After separation by electrophoresis on 1% agarose gels, PCR products were excised from the gel under weak ultraviolet light and purified using the Prep-a-Gene DNA Purification Kit (Bio-Rad Laboratories). The PCR amplicons were a-tailed and cloned using the TOPO XL PCR Cloning™ Kit (Invitrogen) according to the manufacturer's instructions. Electrocompetent cells were electroporated and the transformed cells were plated onto two plates and grown up at 37°C. Several colonies from each isolate were PCR-screened for the presence of the 16S rRNA gene insert. Colonies with inserts of the correct size were then selected, grown in LB medium at 37°C for 1 day, and plasmid DNA was isolated by boiling plasmid mini-prep.

Sequencing and phylogenetic analysis

Four fluorescently tagged primers (Li-Cor Corporation) were used to obtain bidirectional reads covering the 16S rRNA clones. The external primers were the IR-700 tagged T7 Forward (5′-TAATACGACTCACTATAGGG-3′) and IR-800 tagged 13 M Reverse (5′-GGATAACAATTTCA CACAGG-3′). The two internal primers were IR-700 ARC-907F (5′-AAACTTAAAGGAATTGGCGGG-3′) and IR-800 ARC-927R (5′-CCCGCCAATTCCTTTAAGT TT-3′). Sequencing was performed with Epicenter's SequiTherm EXCEL™ II DNA Sequencing Kits-LC, in a Perkin-Elmer thermocycler. An initial denaturation step of 5 min at 95°C was followed by 30 sequencing PCR cycles consisting of 30 s at 95°C, 15 s at 55°C and 60 s at 70°C. Samples were mixed with 5 μl of loading buffer containing formamide, then denatured at 94°C for 1 min, and placed on ice before loading into the Li-Cor 4200 autosequencer. Phylogenetic analysis of the near-complete sequences (without primer sequences) was performed with PAUP4.0Star (Swofford 2000), using the general time reversible (GTR) model with empirically determined nucleotide frequencies, and weighted least square distances as optimality criterion; the branching topology was tested with 200 bootstrap replicates. Primer sequences were excluded from the alignment and the phylogenetic analysis. The following sequences were obtained from GenBank for the phylogeny:

T. hydrothermalis, Z70244; *T. radiotolerans*, AF479013; *T. thioreducens*, AF394925; *T. coalescens* AB107767; *T. celer*, M21529; *T. barossii*, U76535; *T. profundus*, Z75233; *T. guaymasensis*, Y08385; *T. gorgonarius*, AB055123; *T. onnurineus,* DQ167232; *T. kodakarensis*, D38650; *T. peptonophilus,* AJ298871; *T. stetteri*, Z75240; *T.* strain 9°N-7, U47109; *T. marinus*, AF479012; *T.* strain AM4, AJ583507; *T. gammatolerans*, AF479014; *T.* strain Rt3, AF017455; *T. waimanguensis*, AF098975; *T. waiotapuensis*, AF134982; *T. zilligii*, U76534; *T. celericrescens*,

AB107768; *T. siculi*, AJ298870; *T. pacificus*, AB055124; *T. acidaminovorans*, AB055120; *T. atlanticus*, AJ310754; *T. barophilus* U82237; *T. sibiricus*, AJ238992; *T. mexicalis*, Z75218; *T.* strain B1001, AB016298; *T. alcaliphilus*, AB055121; *T. fumicolans*, Z70250; *T. litoralis*, Z70252; *T. aegeaicus*, AJ012643; *T. aggregans*, Y08384.

Pyrococcus cluster*: P. glycovorans*, Z70247; *T. chitinophagus*, X99570; *P. horikoshii*, D87344; *P. abyssi*, Z70246; *P. furiosus*, U20163; *P. woesei*, AY519654; *P. hellenicus*, AJ012642. Genus *Palaeococcus*: *Palaeococcus ferriphilus*, AB019239; *Palaeococcus helgesonii*, AY134472.

The GenBank accession numbers for the new strains are: *Pyrococcus* strain GBD, FJ862775; *P.* strain GIL, FJ862776; *P.* strain C2, FJ949575; *P.* strain MA2.31, FJ862777; *P.* strain MA2.32, FJ862778, *P.* strain MA2.34, FJ862779; *T.* strain GB8.20, FJ862780; *T.* strain 9°N2.21, FJ862781; *T.* strain MA2.27, FJ862782; *T.* strain MA2.28, FJ862783; *T.* strain MA2.29, FJ862784; *T.* strain MA2.33, FJ862785; *T.* 9 N°2.20, FJ862786; *T.* strain A4, FJ862787; *T.* strain B4, FJ862788; *T.* strain 11°N.A5, FJ862789; *T.* strain GB18, FJ862790; *T.* strain B1, FJ862791.

Results and discussion

Guaymas Basin isolates

The serial dilutions from Guaymas Basin samples yielded the highest positives (10^4 cells ml^{-1}) from relatively cool sediment horizons (75°C) closer to the sediment surface, and the lowest (10^1 cells ml^{-1}) from hot samples (105°C) deeper in the sediment column (Fig. 1). Pure cultures were obtained from the most dilute tubes showing growth: strain A4 from the 10^4 dilution of the 75°C sample; strain B4 from the 10^4 dilution of the 85°C sample; and strain C2 from the 10^2 dilution of the 95°C sample. Isolates were also obtained from the lower dilutions, and include strain A2, the 10^2 dilution of the 75°C sample, and strain B1, the 10^1 dilution of the 85°C sample. Step 1 of the deepest dilution series showed some growth, but the enrichment culture did not grow further after 12–24 h of incubation, and subsequent isolation attempts were not successful.

DGGE screening

The screened strains from the culture collection (Table 1) fell into eight DGGE types, groups of strains that could be distinguished by their specific DGGE band position (Fig. 2). All strains showed a single DGGE band each, indicating that they were clonal cultures, and not co-cultures or mixtures of different strains (Teske et al. 1996). DGGE Groups 1 and 2

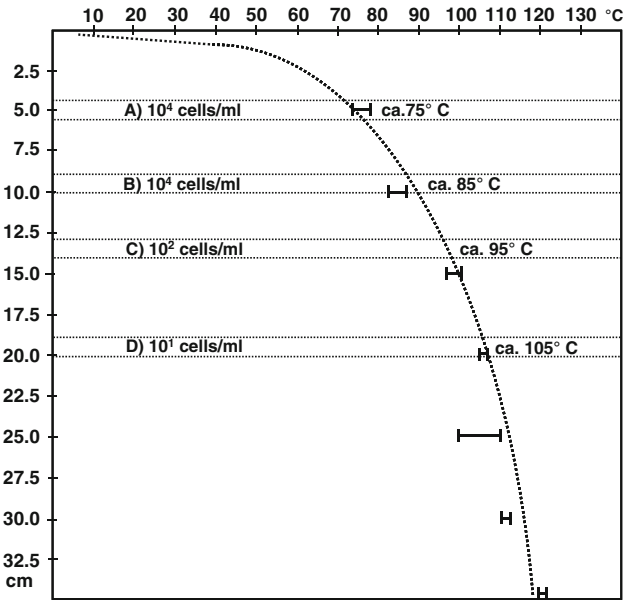

Fig. 1 In-situ temperature gradient and sulfur-reducing archaeal population density of hydrothermally active Guaymas sediments, determined by Alvin temperature probe and shipboard dilution series of sediment core samples (Alvin Dive 3203, Core No. 2, taken near the Everest Mound area in the Southern vent field of Guaymas Basin, sampled 26 April, 1998). The *black line bars* show the measured in situ temperature range for seven sediment horizons; the *stippled lines* show the four sampling horizons for serial dilution. The approximate temperature in each sampling horizon is based on interpolation of the available temperature measurements, spaced in intervals of approx. 5 cm

occurred most frequently and were represented by isolates from multiple different vent sites. Twenty isolates from Guaymas Basin, the Mid-Atlantic Ridge (TAG site), and the East Pacific Rise (9°N and 21°N) belonged to DGGE Group 1, and 15 isolates from Guaymas Basin, the Mid-Atlantic Ridge (Snake Pit) and the East Pacific Rise (21°N) belonged to DGGE Group 2 (Table 1A). The isolates of DGGE Groups 1 and 2 were tested for growth temperature range. Group 1 isolates grew at 90°C but only rarely at higher temperatures, whereas most Group 2 isolates grew also at 96 and 99°C, respectively. Group 2 isolates had been enriched originally at these elevated temperatures, in contrast to Group 1 isolates that had been enriched at 80 or 90°C (Table 1A). In almost all cases, the isolates of DGGE Groups 1 and 2 had been obtained by enrichment without prior dilution. The sole exception was isolate C2, a member of Group 2 from the 1:100 dilution of the 95°C sediment horizon in Guaymas Basin. Enriching from undiluted samples at the same or similar temperatures (90°C for DGGE group 1, 96–100°C for DGGE Group 2) appears to select for relatively few phylotypes within the Thermococcales, even across large geographic distances.

Varying the temperature and the extent of dilution during isolations increased the diversity of the strains

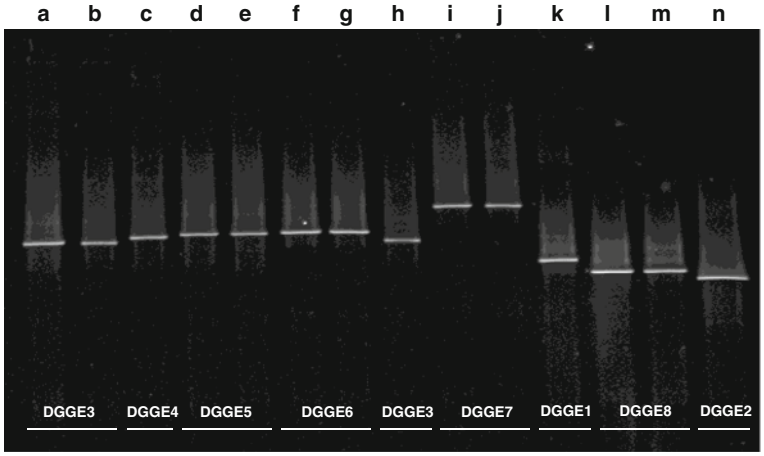

Fig. 2 DGGE patterns of cultured *Thermococcus* and *Pyrococcus* isolates on a 20 to 70% DGGE gel (20% denaturant at the top, 70% at bottom). *Lane a*, Guaymas strain GB18, DGGE type 3. *Lane b*, Guaymas strain GB24, DGGE type 3. *Lane c*, Guaymas strain GB50, DGGE type 4. *Lane d*, East Pacific Rise isolate 11°NA5, DGGE type 5. *Lane e*, East Pacific Rise isolate 11°NVb, DGGE type 5. *Lane f*, Guaymas strain GB128, DGGE type 6. *Lane g*, Guaymas Dilution isolate B4, DGGE type 6. *Lane h*, Guaymas isolate GB18, DGGE type 3. *Lane i*, East Pacific Rise isolate 9°N2.20, DGGE type 7. *Lane j*, Guaymas Dilution isolate A4, DGGE type 7. *Lane k*, Guaymas strain GB20, DGGE type 1. *Lane l*, Guaymas dilution isolate B1, DGGE type 8. *Lane m*, Guaymas dilution isolate A2, DGGE type 8. *Lane n*, Guaymas strain GBD, DGGE type 2

isolated. DGGE Groups 3 to 8 (Fig. 2, Table 1B) consisted of just one or two isolates each, from Guaymas and the East Pacific Rise. The Guaymas dilution strains A2, A4, B1 and B4 grouped with or constituted these less commonly observed clusters, suggesting that isolation by serial dilution and subsequent in situ temperatures, even from a single site and sediment core, yielded increased diversity that was detectable on the DGGE level (Fig. 2). Strains that occur in higher densities in Guaymas Basin are of special interest and may represent microbial populations with new physiological and genotypic adaptations to the hydrothermally heated, petroleum-rich Guaymas sediments.

16S rRNA phylogeny

The near-complete 16S rRNA genes of representative strains from each DGGE group were sequenced in order to identify strains and isolates of the DGGE groups phylogenetically. All DGGE groups were affiliated with the genera *Thermococcus* and *Pyrococcus* (Fig. 3). The DGGE Group 1 consisted of two different phylogenetic clusters within the genus *Thermococcus*, the Mid-Atlantic Ridge strains from the TAG site, and a second cluster of East Pacific Rise strain 9N2.21 and Guaymas strain GB20. DGGE Group 2 consisted of two phylogenetically distinct clusters, isolates from the Mid-Atlantic Ridge (Snake Pit) and Guaymas Basin. Both are closely affiliated with the genus *Pyrococcus*, a monophyletic lineage that differs from *Thermococcus* spp. mainly by higher temperature tolerance (Zillig and Reysenbach 2001). The current delineation between the two genera is becoming consistent with phylogeny, after taxonomic revision and genome analysis of the initially misplaced *Thermococcus* species *T. kodakaraensis* (Atomi et al. 2004; Fukui et al. 2005), a well-studied model system for temperature regulation of gene expression and physiological processes (Fujiwara et al. 2008). A member of the *Pyrococcus* cluster remains misclassified as *T. chitinophagus* (Fig. 3), due to very limited DNA/DNA reassociation and 16S rRNA sequence comparisons in the initial species description (Huber et al. 1995).

The DGGE Groups 3 and 5 were members of a phylogenetically distinct *Thermococcus* lineage (bootstrap support 72%; ca. 4% minimum 16S rRNA sequence distance to other *Thermococcus* and *Pyrococcus* lineages) with the cultured species *T. fumicolans*, *T. aggregans*, and other species from different geographic locations and habitats (Fig. 3). The DGGE Groups 6, 7 and 8 were members of the large polytomic cluster of *Thermococcus* species and strains that include most currently known *Thermococcus* strains and species, and also DGGE Group 1 (Fig. 3). The 16S rRNA sequence of DGGE Group 4 isolate GB50 was fragmentary and insufficient for a reliable phylogenetic placement in a full-length 16S rRNA alignment. Yet, the sequence was distinct from *Thermococcus* strains of other DGGE groups and most similar to *Thermococcus acidaminovorans* (Dirmeier et al. 1998).

DGGE patterns in comparison to 16S rRNA phylogeny

DGGE analyses were performed in order to identify phylogenetically defined groups of *Thermococcus* and

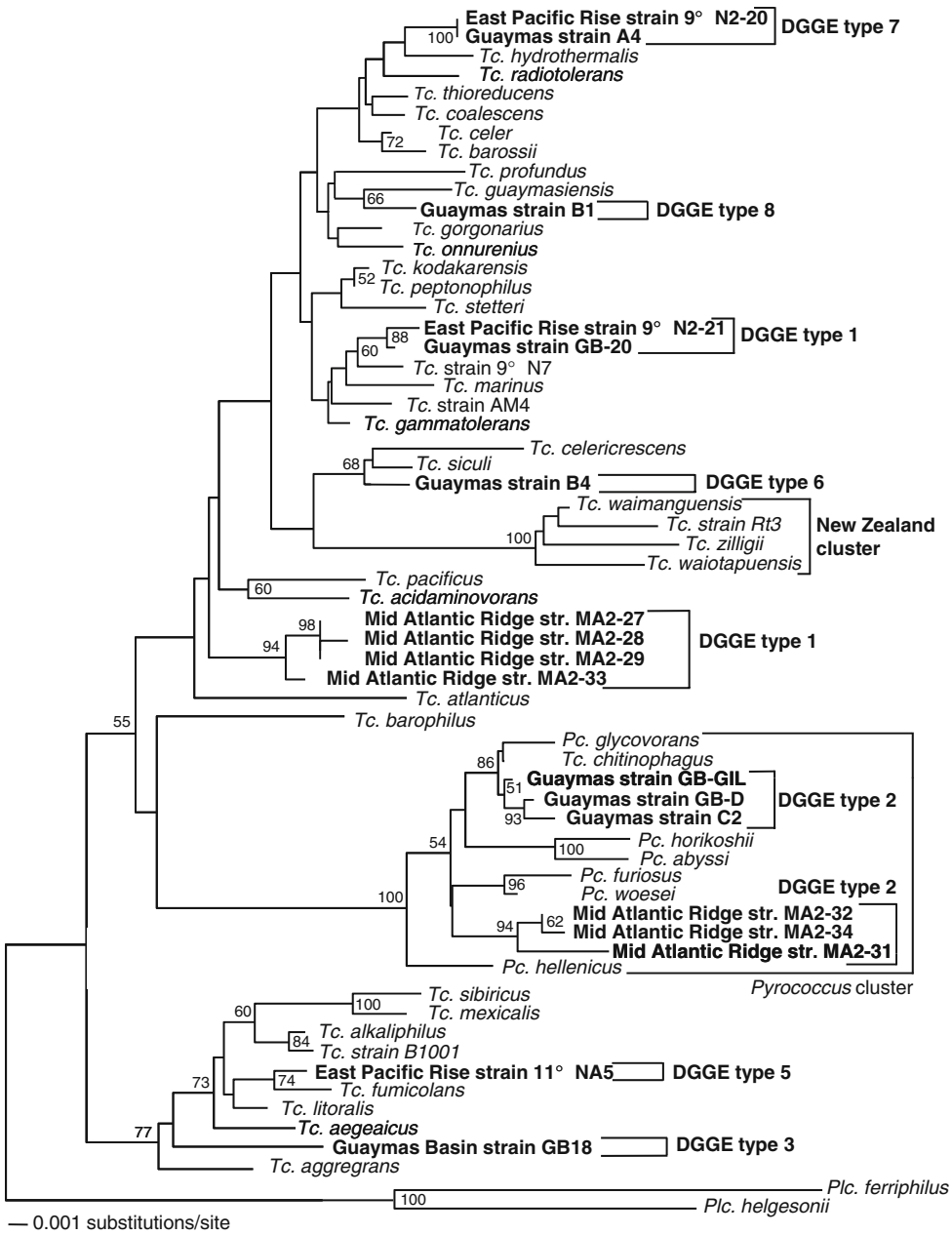

Fig. 3 16S rRNA gene phylogenetic tree of representative Guaymas Basin, East Pacific Rise and Mid-Atlantic Ridge isolates of the genera *Thermococcus* and *Pyrococcus*. DGGE types are the same as in Figs. 1, 2 and Table 1

Pyrococcus strains. As shown by sequencing of full-length 16S rRNA gene amplicons from the same DNA preparations that were used for DGGE, the resulting DGGE groups were mutually exclusive and did not overlap phylogenetically, demonstrating the usefulness of DGGE in phylogenetic sorting of strains and isolates (Fig. 3). The results also show that DGGE is not a substitute for full 16S rRNA gene sequencing. For example, the intragroup differences within DGGE Groups 1 and 2 were only detected by full-length 16S rRNA gene sequencing, which divided the strains in both DGGE Groups into two separate subclusters each (Fig. 3). Most differences in the 16S rRNA gene sequences that account for the subclusters are located in highly variable sequence motifs that lie outside the region of the DGGE fragment, at positions equivalent to *E. coli* positions 341–906, and are therefore not resolvable with this DGGE primer combination. The remaining intragroup mismatches within the strains of DGGE Groups 1 and 2 were almost all on the level of ambiguous nucleotide designations. Sequence mismatches were limited to a maximum of 1 to 3 nucleotide positions per DGGE group, which is close to the resolution limit of DGGE (Muyzer and Smalla 1998).

Substrate requirements

The *Thermococcus* and *Pyrococcus* strains A2, B1, B4, C2, GBD, GB20 and GB18 were checked for growth at 90°C in

mineral medium (TI) amended with different organic substrates (0.4% w/vol). The spectrum of substrates was consistent for all isolates: complex carbon sources, such as yeast extract, peptone, tryptone, casatone and a mixture of 20 amino acids (5–10 mM each) supported growth, as confirmed by culture turbidity and microscopy. No growth was observed on 0.4% w/vol casamino acids, or on carbohydrate polymers (chitin, cellulose and starch), sugars (maltose, glucose), organic acids (benzoate, pyruvate, acetate) and hydrocarbons (toluene, benzene, hexadecane and PCR mineral oil), all at 0.5% w/vol final concentration. The possibility of growth stimulation was tested by adding pyruvate, glucose and maltose to substrate-limited cultures growing on 0.02 and 0.05% peptone, but stimulatory effects were small and variable (maximally a doubling of cell numbers in comparison to peptone-only controls) compared to the effect of increased peptone concentration (0.4% w/vol) which increased cell numbers by one or two orders of magnitude (results not shown).

Diversity patterns

A closer examination of the table of *Thermococcus* and *Pyrococcus* strains reveals how the choice of isolation strategy and temperature influences the diversity of the cultured strains. Five strains were isolated from serially diluted sediment from a single push core at a single geographic location, Guaymas Basin. These strains were enriched at different, near in situ temperatures (A2, A4, B1, B4 and C2) and accounted for four out of eight DGGE Groups (Nos. 2, 6, 7 and 8) and four out of ten 16S rRNA phylotype clusters, since DGGE Groups 1 and 2 consisted of two phylotype clusters each (Fig. 3). Serial dilution combined with enrichment at in situ temperature yields a high diversity of cultured isolates (phylotype/isolate ratio of $4/5 = 0.8$) on a small spatial scale, 20-cm vertical distance within the sediment column of a single push core. In contrast, 20 isolates from diverse sample materials and different geographic origins that were enriched always at 90°C temperature without serial dilution accounted for only two DGGE groups (Nos. 1 and 3) and three out of ten phylotype clusters (phylotype/isolate ratio of $3/20 = 0.15$) (Table 1, Fig. 3). Constant temperature regime and direct enrichment and isolation from source material without prior dilution appears to favor a limited diversity of strains, regardless of the cosmopolitan origin of the sample material from one Atlantic and three Pacific vent sites. Between these two extreme strategies, a third strategy produces an intermediate amount of diverse isolates. Twenty-one strains that were enriched and isolated without serial dilution, but at varied temperature regimes (77, 80, 95, 96, 99 and 100°C) belonged to six different DGGE and phylotype clusters (phylotype/isolate ratio of $6/21 = 0.29$) (Table 1, Fig. 3).

Implications for dispersal

The DGGE groups and phylotype clusters show strong evidence of effective geographic dispersal. For example, DGGE Group 7 harbors a Guaymas Basin and a 9°N East Pacific Rise *Thermococcus* strain with identical 16S rRNA gene sequences (Fig. 3). Similar cases of closely related but spatially separated vent microbial populations are well documented, even on the level of intraspecies diversity tested by DNA/DNA hybridization for Atlantic and Pacific strains of the bacterial sulfur oxidizer *Thiomicrospira* (Wirsen et al. 1998). The classical explanation is effective dispersal by megaplumes after massive hydrothermal eruptions. Large quantities of erupting hydrothermal fluids and gasses can reach heights of 1–2 km and remain detectable for months. After dilution with seawater, these water masses are only minimally warmer than the deep-water background, and are fully oxygenated; yet some vent archaea survive in this environment for several weeks or months, facilitating dispersal (Summit and Baross 1998). Less dramatic and episodic forces would also suffice to distribute *Thermococcus* and *Pyrococcus* across wide distances. Deep-sea bottom currents with a speed of 0.05 to 1 m/s can cover 43 to 864 km in 10 days, and could carry microbial cells. Laboratory experiments have shown that some anaerobic vent hyperthermophilic archaea can survive exposure to cold, oxygenated seawater for at least two weeks (Jannasch et al. 1992).

Spatial scale of microbial activity in Guaymas sediments

In the special case of the 1998 Guaymas Basin isolates, the sampling context (sediment depth, temperature gradient and cell density of cultivable Thermococcales) is known, and contributes to a partial habitat characterization that is consistent with other studies. The distribution of cultivable *Thermococcus* and *Pyrococcus* isolates in hydrothermally active Guaymas sediments is strongly skewed toward the upper 10-cm layer. The highest concentration of cultivable cells (10^4 cells/ml) was found in the upper 10 cm at temperatures up to 85°C, decreased to 10^2 cells/ml at 13-cm depth and temperatures of ca. 95°C, and approached the detection limit at 20 cm depth and ca. 105°C where an enrichment on undiluted sediment showed initial growth but did not yield any isolates (Fig. 1). Other studies have found a similar preference of different groups of microorganisms for the surficial sediments at Guaymas. Bacterial and archaeal lipids are most abundant in the surface 5 cm layer (Guezennec et al. 1996; Teske et al. 2002), and decrease by two to three orders of magnitude toward 10–15 cm depth (Schouten et al. 2003). DNA for PCR amplification, cloning and sequencing of bacterial and

archaeal 16S rRNA genes and functional genes for methanogenesis and sulfate reduction (*mcrA*, *dsrAB*) could be extracted from max. 7 cm (*dsrAB*) to 15 cm (*mcrA*) sediment depth (Teske et al. 2002; Dhillon et al. 2003, 2005). Shipboard measurements of sulfate reduction rates in *Beggiatoa*-covered Guaymas Basin sediments showed the highest rates in surface sediments (upper 6 cm) at mesophilic or moderately thermophilic temperatures (Weber and Jørgensen 2002). The depth of the microbially active surficial sediment layer depends on locally variable hydrothermal fluid and heat flow; temperature appears to be the most important control factor that limits microbial life to the surficial sediment layers at Guaymas Basin.

Ecophysiological aspects of Thermococcales diversity

Related cultured *Thermococcus* and *Pyrococcus* species differ in temperature sensitivity, substrate spectra and proteolytic repertoire (Zillig and Reysenbach 2001). The physiological differences among *Thermococcus* and *Pyrococcus* species and strains, and their numerous genotype and 16S rRNA variants detected in this study and in other cultivation surveys (Marteinsson et al. 1995; Lepage et al. 2004; White et al. 2008), indicate that this diversity is a consistently recurring key feature of archaeal vent communities and therefore must have an ecological explanation. Resource partitioning, for example by different hydrolytic enzyme repertoire, temperature range and growth rate (Holden et al. 2001), different degrees of toxicity tolerance (Edgcomb et al. 2004) and growth in surface-attached biofilms surrounded by capsular exopolysaccharides (LaPaglia and Hartzell 1997; Rinker and Kelly 1996; Schrenk et al. 2003) are the likely adaptive strategies that sustain and maintain Thermococcales diversity within a complex mosaic of physically and chemically different hydrothermal vent microenvironments (Reysenbach and Shock 2002). A fuller understanding of *Thermococcus* and *Pyrococcus* diversity and function in their in situ hydrothermal vent context might require in situ colonization and biofilm formation experiments coupled with strain-specific molecular monitoring of ecophysiologically divergent *Thermococcales* strains.

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