

# Diversity among three novel groups of hyperthermophilic deep-sea *Thermococcus* species from three sites in the northeastern Pacific Ocean

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## Abstract

Eight new strains of deep-sea hyperthermophilic sulfur reducers were isolated from hydrothermal vent fields at 9°50'N East Pacific Rise (EPR) and at the Cleft and CoAxial segments along the Juan de Fuca Ridge (JdFR). 16S rRNA gene sequence analysis showed that each strain belongs to the genus *Thermococcus*. Restriction fragment length polymorphism patterns of the 16S/23S rRNA intergenic spacer region revealed that these isolates fell into three groups: those from the EPR, those from fluid and rock sources on the JdFR, and those isolated from *Paralvinella* spp. polychaete vent worms from the JdFR. The optimum-temperature specific growth rates and the temperature ranges for growth were significantly higher and broader for those strains isolated from worms relative to those isolated from low-temperature diffuse hydrothermal fluids. Furthermore, the worm-derived isolates generally produced a larger array of proteases and amylases based on zymogram analyses. The zymogram patterns also changed with growth temperature suggesting that these organisms alter their lytic protein suites in response to changes in temperature. This study suggests that there is significant phenotypic diversity in *Thermococcus* that is not apparent from their highly conserved 16S rRNA nucleotide sequences. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Phylogeny; Intergenic spacer region; Deep-sea hydrothermal vent; *Thermococcus*; *Paralvinella*

## 1. Introduction

Hyperthermophiles are defined as those microorganisms with optimal growth temperatures above 80°C or as those that grow at temperatures exceeding 90°C [1,2]. Among hyperthermophiles, the genus *Thermococcus* contains the highest number of characterized species [1]. *Thermococcus* spp. have been found in shallow marine thermal springs, deep-sea hydrothermal vents [1,2], oil reservoirs [3–7], and freshwater thermal pools in New Zealand [8,9]. *Thermo-*

*coccus* spp. are all anaerobic heterotrophs that ferment peptides and sugars, their growth is stimulated through sulfur reduction, and optimum growth occurs between 76 and 88°C. The 16S rRNA nucleotide sequences of *Thermococcus* spp. are highly conserved, yet there is often very little DNA homology between species [10,11] and their G+C mol% range from 38 to 60%. *Thermococcus litoralis* and *Thermococcus celer* differ by less than 3% in their 16S rRNA gene sequences, yet the G+C mol% of the strains are 38 and 56.6%, respectively, with only 10% DNA homology between the two strains [12]. Therefore, though the genus *Thermococcus* is functionally and phylogenetically coherent, there is potential for phenotypic diversity between species.

Eight strains of *Thermococcus* spp. were isolated from three geographically distinct, deep-sea hydrothermal vent sites in the northeastern Pacific Ocean and from different source materials. These isolates were purified and cultured in the same medium, transferred at the same times, and incubated together for comparative analyses. To assess the

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diversity within this group of isolates, the strains were compared using 16S rRNA gene sequence alignments, polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analyses of the 16S/23S rRNA intergenic spacer (IGS) region, G+C mol%, growth rate measurements at various temperatures, and protease and amylase patterns in substrate-containing polyacrylamide gels (i.e. zymograms). These phylogenetic and phenotypic data were then correlated with geographic location of isolate origin and habitat type.

## 2. Materials and methods

### 2.1. Growth medium

The organisms were grown in either 10 ml of medium within a 25-ml Balch tube or in 250 ml of medium within a 500-ml serum bottle, each sealed with a butyl rubber stopper. The medium was prepared as described previously [13]. Medium components were prepared as separate sterile stock solutions and stored at room temperature. Stock solutions were as follows: 1× base salts solution, containing, per liter, 19.6 g of NaCl, 3.3 g of Na<sub>2</sub>SO<sub>4</sub>, 0.5 g of KCl, 0.05 g of KBr, 0.02 g of H<sub>3</sub>BO<sub>3</sub>, and 8.8 g of MgCl<sub>2</sub>·6H<sub>2</sub>O; 100× trace minerals solution, containing, per liter, 0.01 g of CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.1 g of ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.005 g of CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.2 g of MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.1 g of Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.05 g of KBr, 0.05 g of KI, 0.1 g of H<sub>3</sub>BO<sub>3</sub>, 0.05 g of NaF, 0.05 g of LiCl, 0.05 g of Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, 0.01 g of NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.005 g of VOSO<sub>4</sub>·2H<sub>2</sub>O, 0.005 g of H<sub>2</sub>WO<sub>4</sub>, 0.005 g of Na<sub>2</sub>SeO<sub>4</sub>, 0.005 g of SrCl<sub>2</sub>·6H<sub>2</sub>O, and 0.005 g of BaCl<sub>2</sub>; 1% CaCl<sub>2</sub>·2H<sub>2</sub>O solution (200×, containing 10 g per liter); 100× N–P mixture, containing, per liter, 43.0 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 60.5 g of NaNO<sub>3</sub>, and 3.6 g of KH<sub>2</sub>PO<sub>4</sub>; 500× Fe EDTA solution, containing, per liter, 1.54 g of FeSO<sub>4</sub>·7H<sub>2</sub>O and 2.06 g of Na-EDTA; and resazurin solution at 0.2 g per liter.

Each medium (YPS) was composed of 1× base salts solution containing, per liter, 10 ml of 100× trace minerals solution, 5 ml of 1% CaCl<sub>2</sub>·2H<sub>2</sub>O, 10 ml of 100× N–P mixture, 2 ml of 500× Fe EDTA solution, 5 ml of resazurin solution, 1 g of 2-(*N*-morpholino)ethanesulfonic acid (Sigma), 3 g of yeast extract (Difco), 3 g of peptone (Difco), and 10 g of elemental sulfur (JT Baker). The pH (measured at room temperature) was adjusted to 6.0 with 1 N NaOH. After adding the medium, the headspace of the sealed bottles was flushed with argon. Residual oxygen was removed from the medium by adding 0.05% Na<sub>2</sub>S·9H<sub>2</sub>O.

### 2.2. Enrichment and purification of deep-sea isolates

Hyperthermophilic sulfur reducer enrichments were made from hydrothermal vent samples collected by the research submarine *Alvin* in 1993 and 1994. The samples

were from two deep-sea hydrothermal vent sites along the southern Juan de Fuca Ridge (JdFR) (CoAxial and Cleft segments) in the northeastern Pacific Ocean and from one site on the East Pacific Rise (EPR) at 9°50'N latitude (Fig. 1). The samples from the CoAxial segment were collected from the 'Floc' and the 'Source' vents sites, which are 15 km apart. The Floc site consisted of low-temperature hydrothermal fluids venting through cracks in the seafloor. Samples from the Source site were collected from two hydrothermal sulfide rock deposits that were 10 m apart. Sulfur reducers were isolated from sulfide rock and vent polychaete worm (*Paralvinella sulfincola* and *Paralvinella palmiformis*) samples collected at the base of the Source site deposits and from the fluids at the Floc site (Table 1). Samples from the Cleft segment were collected from two hydrothermal sulfide rock deposits that are 1 km apart. Sulfur reducers were isolated from *P. sulfincola* and *P. palmiformis* worms that lived on the outer surface of 'black smoker' chimneys on the deposits (Table 1). Sulfur reducers were isolated from low-temperature fluids venting from a crack and from a sulfide rock sample at 9°N EPR that were 1 km apart (Table 1).

These samples were used to inoculate YPS medium, which were then incubated at 90°C at sea in enclosed, oven-heated sandbaths. Enrichments showing growth were turbid after 12–36 h of incubation. To obtain pure cultures, the 'dilution-to-extinction' technique [13] was employed and repeated twice at 90°C using silicon oil baths on land. Eight of the enrichments were selected and used for further study. *T. litoralis* (DSM 5473), *Pyrococcus furiosus* (DSM 3638) and *Pyrococcus* sp. strain JdF2 from the Endeavour segment hydrothermal vent field along the northern JdFR (Fig. 1) were included in the study for comparative purposes.

### 2.3. 16S rRNA gene nucleotide sequencing

DNA was extracted from cell pellets using IsoQuick kit (Orca Research). The universal archaeal primers 21Fa (TTCCGGTTGTACCYGCCGGA) and 958Ra (YCCG-GCGTTGAMTCCAATT) were used for DNA amplification using PCR as described previously [14]. The amplification profile began with a 1-min initial denaturation at 95°C, followed by 30 cycles of 20 s at 90°C (denaturation), 30 s at 55°C (annealing), and 90 s at 72°C (extension) ending with a final extension of 5 min at 72°C. The PCR products were sent to the Molecular Genetics and Instrumentation Facility at the University of Georgia and both strands were sequenced on an Applied Biosystems sequencer. The PCR products were ~900 bp long, and the entire length of the sequences was aligned with all known members of the Thermococcales (sequences obtained from the GenBank and EMBL databases) using tools provided by the Ribosomal Database Project [15]. Alignments were finished by eye with careful attention to secondary structure and variable regions. Evolutionary

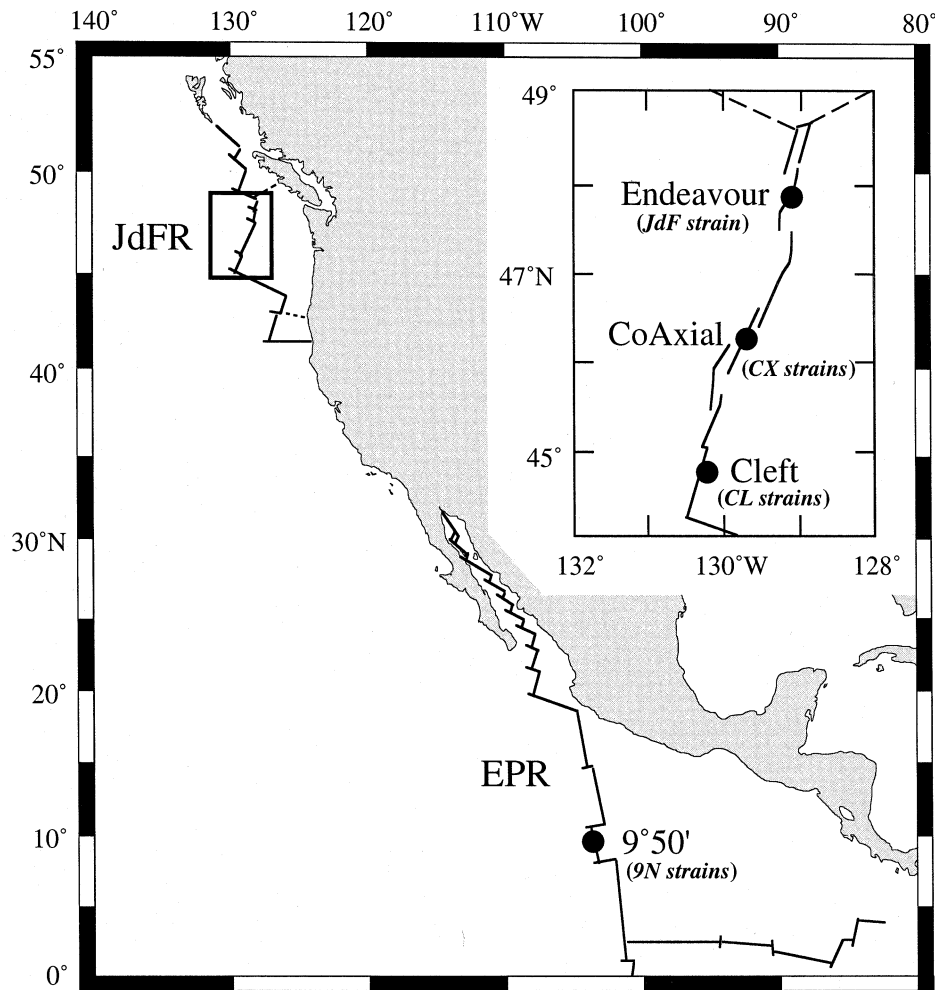


Fig. 1. Map of the eastern North Pacific Ocean showing the JdFR and the 9°50'N hydrothermal vent site along the EPR. The boxed inset is an enlargement of the JdFR that shows the locations of the Cleft, CoAxial, and Endeavour segment hydrothermal vent fields.

models were tested on each data set, with a likelihood ratio test [16] performed to determine the simplest model appropriate for the data. The most appropriate model of evolution for this data set was the Tamura–Nei three parameter model [17] and the data were best modeled with an estimated proportion of sites remaining invariant and gamma-distributed rate variations between the remaining sites [18]. This model was used to construct minimum-evolution distance trees with PAUP v.4.0b4a (D.L. Swofford, Sinauer Assoc.), and the parameters for the evolutionary models were estimated using a maximum likelihood evaluation of the trees. This procedure was iterated three times after which a heuristic search with these final parameters and the minimum-evolution criteria including 1000 random-addition replicates generated the final tree. Five hundred bootstrap replicates [19] were also performed using these parameters. Trees were visualized with TreeView [20]. These fragments of the 16S rRNA sequence for the isolates have been submitted to GenBank and assigned accession numbers AF350363 (9N2), AF350364 (9N3), AF350365 (CX1), AF350366 (CX2),

AF350367 (CX3), AF350368 (CX4), AF350369 (CL1), and AF350370 (CL2).

#### 2.4. RFLP analysis of the 16S/23S rRNA IGS region

DNA was extracted from each strain, and the IGS region between the 16S and 23S rRNA genes was analyzed by PCR-RFLP as described previously [21,22]. Primers 1406F (TGCACACACCGCCCGT) and 213aR (GTTGGTTTCTTTTCCT) were used for DNA amplification of the IGS region using PCR [14]. The amplification profile began with a 3-min initial denaturation at 95°C, followed by 30 cycles of 1.5 min at 95°C (denaturation), 1 min at 48°C (annealing), and 2 min at 72°C (extension) ending with a final extension of 5 min at 72°C. Amplified products were separated by electrophoresis in 1% agarose, stained for 40 min with SYBR green (Molecular Probes), and analyzed with a fluorometric imager (Fluor Imager 575, Molecular Dynamics) for size determination. Five hundred ng of PCR product was digested separately with the restriction endonucleases *HhaI*

Table 1  
Sample locations, sources materials, and phenotypic and phylogenetic characteristics of the new *Thermococcus* strains, *T. litoralis*, *Pyrococcus* strain JdF2, and *P. furiosus*

Strain	Sampling location and date	Source	$T_{\text{range}}$ (°C) ( $T_{\text{opt}}$ (°C))	$k_{\text{max}}$ ( $\text{h}^{-1}$ )	G+C (mol%)	<i>HhaI</i> RFLP pattern (bp)	<i>MspI</i> RFLP pattern (bp)	Protease pattern <sup>c</sup> (kDa)	Amylase pattern <sup>c</sup> (kDa)
CX1	CoAxial segment, JdFR, 1993	18°C diffuse hydrothermal fluid	65–92 (87)	0.97	48.9	450, 125	205, 125, 110	ND	(200, 180, 90)
CX2	CoAxial segment, JdFR, 1993	278°C 'black smoker' hydrothermal vent fluid	55–92 (87)	1.22	47.4	450, 125	205, 125, 110	ND	(90)
CX3	CoAxial segment, JdFR, 1993	284°C 'black smoker' hydrothermal vent fluid	55–92 (87)	1.04	48.6	450, 125	205, 125, 110	[110,105], 70, [60], [55]	(200, 180, 120, 90), 55, 48
CX4	CoAxial segment, JdFR, 1994	Alvinellid worm from a 'black smoker' sulfide	55–94 (88)	1.96	50.0	450, 125	205, 110, 95, 70, 55	110, 100, 95, 70, (55)	210, 160, (120), 90, 37, 34
CL1	Cleft segment, JdFR, 1994	Alvinellid worm from a 'black smoker' sulfide	55–94 (88)	1.67	51.6	450, 125	205, 110, 95, 70, 55	(110, 105), 70	210, 160, 90, 37, 34
CL2	Cleft segment, JdFR, 1994	Alvinellid worm from a 'black smoker' sulfide	55–94 (88)	2.40	49.6	450, 125	205, 110, 95, 70, 55	100, 70, 65, 60, [55], (45), (37, 34)	210, 100, 37, 34
9N2	9°N EPR, 1993	'black smoker' sulfide 30°C diffuse vent hydrothermal fluid	60–92 (87)	0.78	49.1	230, 205, 125	125, 110, 90, 80	$\geq 100$ , ( $\geq 55$ )	[110], 75
9N3	9°N EPR, 1994	Sulfide rock material from a 'black smoker' structure	55–97 (88)	1.58	52.9	230, 205, 125	125, 110, 90, 80	ND	ND
<i>T. lit</i> <sup>a</sup>	Lucrino, Bay of Naples, Italy	Shallow-water geothermally heated marine sediments	55–94 (88)	0.85	37.4	220, 125	210, 145, 105	ND	110, 70
JdF2	Endeavour segment, JdFR, 1991	Alvinellid worm from a 'flange' sulfide outcrop	70–103 (96)	1.03	39.2	230, 220, 130	130, 75, 60	200, $\geq 55$	ND
<i>P. fur</i> <sup>b</sup>	Porto di Levante, Vulcano, Italy	Shallow-water geothermally heated marine sediments	70–103 (100)	1.12	36.3	ND	ND	$\geq 65$	[200], 120, 55, (52, 48)

ND = not detected;  $k_{\text{max}}$  = maximum specific growth rate; bp = length in base pairs.

<sup>a</sup>From Neuner et al. [12].

<sup>b</sup>From Fiala and Stetter [38].

<sup>c</sup>Brackets indicate protein was only found at optimum growth temperature; parentheses, only at minimum growth temperature.

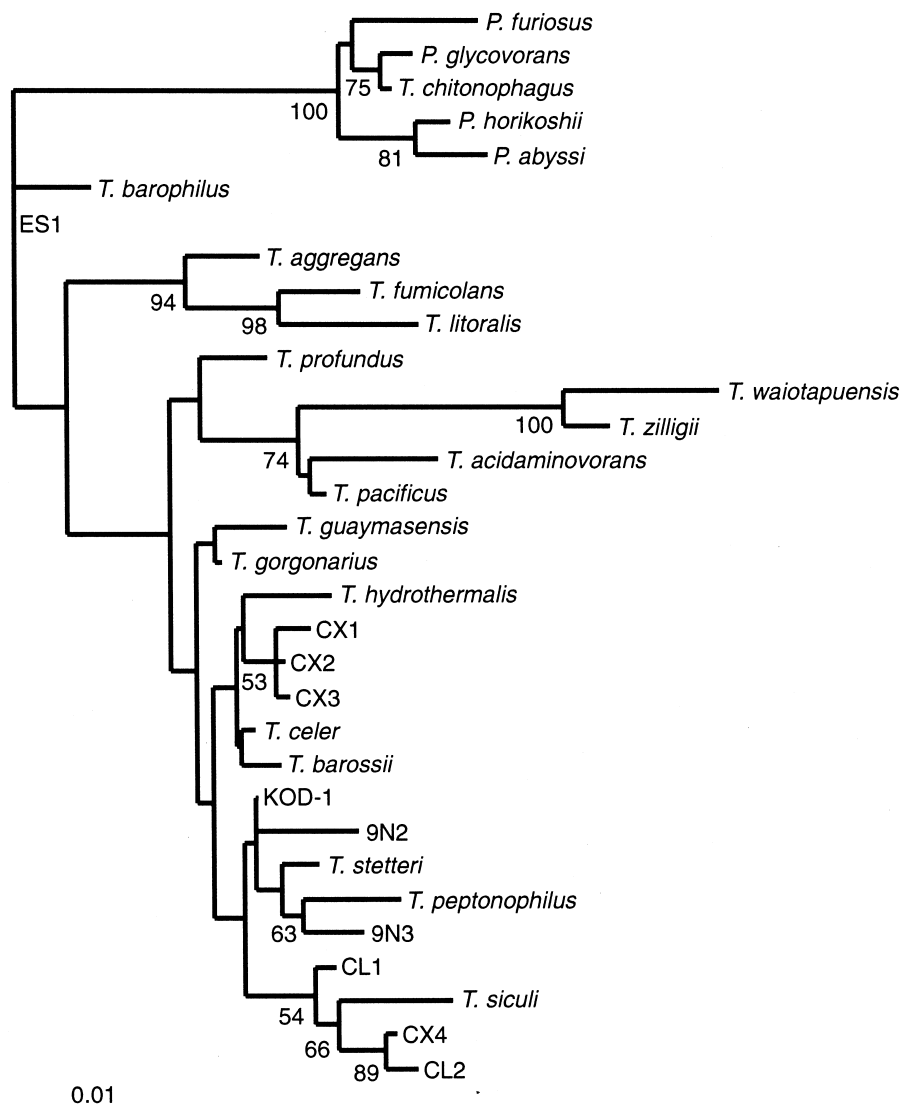


Fig. 2. Phylogenetic tree showing the relationship between the eight new isolates of this study and other closely related hyperthermophilic Archaea. Branch lengths are proportional to genetic distances (scale bar represents one nucleotide substitution per 100 bp) and numbers at the nodes are relevant bootstrap values (see text).

and *MspI*. The digests were separated by electrophoresis in 4% MetaPhor agarose (FMC BioProducts), stained for 90 min with SYBR green, and analyzed with a fluorometric imager.

### 2.5. Genomic G+C mol%

The genomic G+C mol% was determined for each strain as described previously [23]. Each DNA sample was diluted to 0.75 µg per ml in 15 mM NaCl–1.5 mM sodium citrate (pH 7.0). DNA concentrations and purities were determined by absorbance measurements at 260 and 280 nm wavelength. Duplicate samples were heated from 45 to 100°C at a rate of 1°C per min using a PTP-6 temperature programmer (Peltier) while their absorbance was measured spectrophotometrically at 260 nm (Perkin Elmer). The

melting temperature of the DNA was determined by taking the first derivative of the melting curve (PECSS software, Perkin Elmer). The G+C mol% of each DNA sample was calculated using *Escherichia coli* as a reference [24].

### 2.6. Temperature growth ranges and growth rates

Growth curves were generated from duplicate cultures of each strain incubated at temperatures spanning the growth range of the organisms. Subsamples from different time points were preserved in glutaraldehyde (type II, 1% final concentration), filtered onto 0.2-µm pore-size membrane filters prestained with Irgalan black, stained with 4',6-diamidino-2-phenylindole [25], and counted with an epifluorescence microscope. The specific growth rates (*k*)

were calculated by linear regression analysis from the exponential portions of the growth curves.

### 2.7. Zymogram analyses of hyperthermophilic proteases and amylases

Proteins were extracted from each strain as described previously [26]. Cultures were grown at their optimum and minimum growth temperatures and harvested approximately two doublings prior to reaching their stationary phase cell concentration ( $\sim 5 \times 10^7$  cells ml<sup>-1</sup>). The cultures were filtered through an 11.0- $\mu$ m retention filter to remove particulate sulfur. The filtrate was centrifuged at  $10\,000 \times g$  for 30 min at 4°C. The pelleted cells were resuspended and centrifuged three times in 50 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5). The cells were then resuspended in the phosphate buffer, ruptured by shaking with 0.1-mm diameter glass beads, and spun at  $16\,500 \times g$  for 5 min. The protein concentration of the supernatant was determined using the Bradford method [27].

The procedures used to detect protease and amylase activities in substrate gels were based on methods described previously [28–30]. Equal protein concentrations were loaded onto glycine-buffered 0.1% SDS–8% polyacrylamide gels containing either 0.3% (w/v) gelatin or 0.3% starch for protease or amylase zymograms, respectively. After electrophoresis, the substrate gel was washed for 1 h at room temperature in 50 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5) containing 2.5% (v/v) Triton X-100 and then incubated in phosphate buffer only for 3 h at 85°C. For detection of protease activity, the gelatin gels were stained in a solution containing 1.8 g Amido black and 0.04 g Coomassie brilliant blue R-250 per liter of 35% methanol–7% acetic acid for 1 h and then destained in the methanol–acetic acid solution. For detection of amylase activity, the starch gels were stained in a solution containing 0.15% I and 1.5% KI for 2 min. Clear bands in the stained gels identified the locations of lytic enzymes. Protein size markers added to one lane of the protease gel were visible with the Coomassie staining. Protein size markers were loaded onto

an amylase gel, separated in tandem with samples loaded onto a separate experimental amylase gel, and stained with Coomassie to determine the sizes of the starch-degrading proteins.

## 3. Results

### 3.1. DNA analyses

All eight of the isolates were *Thermococcus* spp. based on phylogenetic analysis of the 16S rRNA sequences (Fig. 2). Amplification of the IGS region yielded one 600-bp product for each isolate, which is typical for *Thermococcus* and *Pyrococcus* spp. [21,31]. Restriction enzyme digestion of the product yielded two distinct RFLP patterns for *HhaI* and three for *MspI* (Fig. 3, Table 1), dividing the isolates into three groups: group I, strains 9N2 and 9N3; group II, strains CX1, CX2, and CX3 (from fluids and sulfides); and group III, strains CL1, CL2, and CX4 (from vent worms). Group I strains, from 9°N EPR, had RFLP patterns distinct from the JdFR strains in groups II and III. Groups II and III contain the same *HhaI* restriction sites but are distinguished by *MspI*. The G+C mol% of the new strains were not significantly different (Table 1) and ranged between 47.4 and 52.9%, but all differed from *T. litoralis*, which had a G+C mol% of 37.4%.

### 3.2. Temperature growth ranges and growth rates

All of the isolates had an optimum growth temperature of 87 or 88°C (Table 1) and appeared phenotypically to be *Thermococcus* spp. The growth temperature ranges for 9N2 and CX1 (the two diffuse-fluid strains) were 5 and 10°C narrower, respectively, than those of the other six strains (Fig. 4, Table 1). The three strains from alvinellid worms (group III: CX4, CL1, and CL2) showed a rapid increase in their growth rate at their optimum growth temperature while the growth rates of the diffuse-fluid strains (CX1 and 9N2) remained relatively low at their

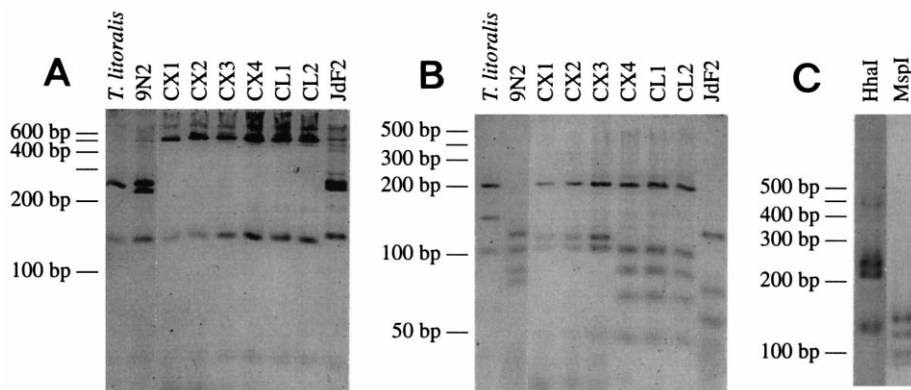


Fig. 3. 16S/23S IGS region RFLP patterns of the isolates (except 9N3) using the *HhaI* (A) and *MspI* (B) restriction enzymes. (C) The RFLP patterns for strain 9N3 using the restriction enzymes *HhaI* (left lane) and *MspI* (right lane). Base pair lengths are indicated on the left side of the figures.

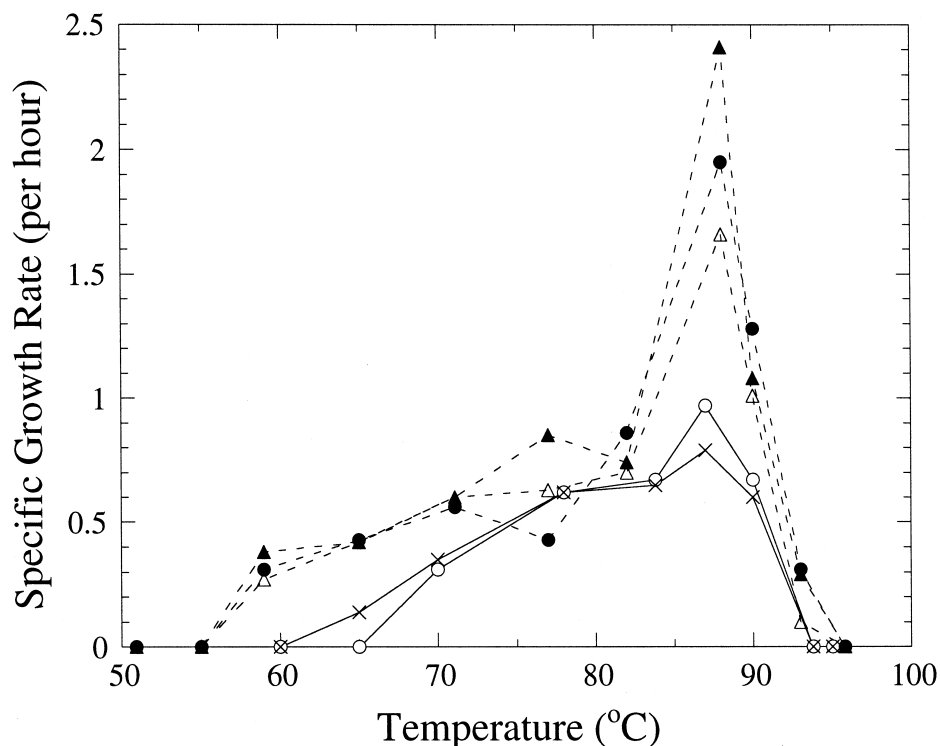


Fig. 4. The specific growth rates of the *Thermococcus* strains from the *Paralvinella* spp. polychaete worms (dashed lines) and from the diffuse hydrothermal fluids (solid lines) over their temperature ranges for growth. The strains shown are 9N2 (X), CX1 (○), CX4 (●), CL1 (△), and CL2 (▲).

optimum. The optimum-temperature growth rates of the strains from the alvinellid worms differed significantly from the growth rates of the diffuse-fluid vent strains ( $P < 0.05$ ; ANCOVA followed by a Tukey multiple range test) with the exception of CX1 and CL1 where the difference was weakly significant ( $P < 0.1$ ). The specific growth rates of the strains isolated from sulfides (data not shown) ranged between those of the other two groups and did not differ from either ( $P > 0.1$ ).

### 3.3. Comparisons of protease and amylase compositions

The protease (Fig. 5A) and amylase (Fig. 5B) zymogram patterns demonstrated significant phenotypic diversity in these strains, while growth temperature influenced the kind and number of proteases and amylases produced. Strains CX1, CX2, 9N2 and *T. litoralis* did not produce detectable proteases, while the other strains generally produced proteases with molecular masses of 100–110 kDa, 65–70 kDa, and 55 kDa. The extended clearing observed for the *P. furiosus*, JdF2, and 9N2 samples was observed previously for *P. furiosus* [28]. The result indicates that certain protease(s) were active at room temperature in the presence of SDS during electrophoresis. JdF2 and 9N3 were the only strains that did not produce detectable amylases. Strains CL1, CL2, and CX4 (group III, all from alvinellid worms) consistently produced amylases with masses of 210 kDa, 160 kDa, 90–100 kDa, and 35–40 kDa. Strains CL1, CL2, 9N2, and CX4 produced a differ-

ent array of proteases when grown at minimum temperature (Fig. 5A), while strains CX1, CX2, and CX3 produced different arrays of amylases at minimum temperature (Fig. 5B).

## 4. Discussion

One of the most thoroughly studied groups of hyperthermophiles is the order Thermococcales (*Pyrococcus* and *Thermococcus* spp.). *Thermococcus* spp. have been isolated or detected in a wide range of geothermal habitats [1–9]. *Thermococcus* spp. appear to have especially slow evolving 16S rRNA genes relative to the whole genome [10–12]; therefore, the 16S rRNA gene may not be a good measure of overall genetic or phenotypic diversity within this genus. Our study suggests genetic and phenotypic diversity in our strains that is not apparent in their 16S rRNA sequence differences. Our strains appear to fall into three groups of organisms that correlate with their sample location and source materials, which suggests that they are adapted to specific environments. This adaptation may include not only divergences based on geographic separation, but also physiological and biochemical adaptations to specific biotopes.

The eight hyperthermophilic strains isolated in this study fall into three distinct groups based on genetic and phenotypic characteristics. Groups I (9N2, 9N3), II (CX1, CX2, CX3), and III (CL1, CL2, CX4) can be distinguished

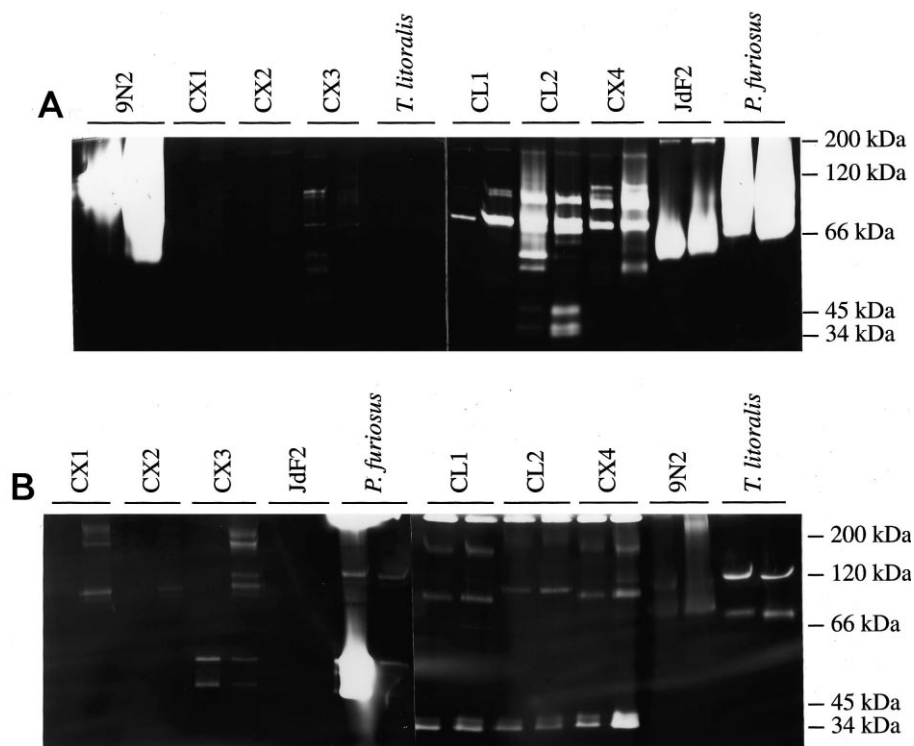


Fig. 5. Protease (A) and amylase (B) zymogram patterns for the isolates when grown at their optimum (left lane) and minimum (right lane) growth temperatures. The low-temperature samples from CX2, CX3, CX4, CL1, CL2, 9N3, and *T. litoralis* were from cultures incubated at 60°C, 9N2 was incubated at 65°C, and CX1, *Pyrococcus* strain JdF2, and *P. furiosus* were incubated at 70°C. Molecular masses are indicated on the right side of the figures.

by analysis of the IGS-RFLP patterns (Fig. 3). Phylogenetic analysis of the 16S rRNA gene (Fig. 2) is consistent with these groupings, though the 16S rRNA gene sequence similarity between these isolates (98–100%) is too high to make significant distinctions between them.

Raguénes et al. [32] reported genotypic diversity within similar hyperthermophile strains from hydrothermal vent sites along the EPR and predicted associated phenotypic diversity. This appears to be true for the *Thermococcus* strains described in this study. The group III isolates are the most phenotypically distinct, as they have significantly different maximal growth rates, they exhibit a qualitatively different (i.e. non-Arrhenius) response to temperature, and they generally produce a larger suite of lytic enzymes relative to the other two groups for the nutritional conditions used in this study. The rapid maximal growth rates of group III strains stand out in the genus *Thermococcus*; the only other *Thermococcus* sp. with a comparable growth rate is *T. guaymasensis* [33]. Group I and II strains, while obviously genetically different groups, were not distinguished by the phenotypic tests of this study.

By studying the diversity of *Thermococcus* spp., we may understand how these organisms have diverged from one another, either through geographic isolation or in response to changing environmental conditions. Our results indicate that *Thermococcus* genetic diversity can be correlated with geographic location and habitat. A broad biogeographical distribution pattern is suggested by the geographical divi-

sion of group I strains (9°N EPR) from groups II and III (JdFR). Distinct sample types, reflecting different habitats, distinguish group II from group III isolates.

If organisms have adapted to a specific habitat, those habitat characteristics should be reflected in their physiology. The group III strains were derived from *Paralvinella* spp. vent polychaete worms and appear to be well-adapted to living in a worm-influenced habitat. *P. sulfincola* and *P. palmiformis* colonize the surfaces of black smoker sulfide structures that emit fluids up to 400°C, create tubes that penetrate into the steep, unstable thermal gradient found in sulfides, and deposit copious organic compounds and elemental sulfur in a mucous [34]. *Thermococcus* strains CL1, CL2, and CX4, which were isolated from the worms, generally produced the largest number of proteases (Fig. 5A) and their amylase patterns were unique compared to those of groups I and II (Fig. 5B). The production of large suites of lytic enzymes should allow utilization of worm-derived organic compounds. This organic-rich growth substrate may also select for the high maximal growth rates seen in group III isolates. *T. guaymasensis*, with comparable growth rates, was isolated from Guaymas Basin [33], which contains 400-m-thick sediments that are rich in organics and petroleum [35].

Not only did group III strains have the highest maximal growth rates, they also had the broadest temperature ranges for growth, perhaps as a means of optimizing growth in an environment of steep thermal gradients and

sharp temperature fluctuations. The temperature at the anterior end of alvinellid worms was measured in situ at 22°C while that at the posterior end of the worms, 4–8 cm away, was 81°C [36]. The group III isolates varied the suites of proteases they produced as growth temperature changed (Fig. 5A), which may enhance their ability to recover protein for growth within the thermal gradients of the worm environment. Enzymes produced at the minimum growth temperature are potentially important for substrate return from neighboring organic-rich environments with temperatures below the growth range of the organism.

*Thermococcus* spp. appear to be ubiquitous in marine thermal habitats. Furthermore, the relative isolation of hydrothermal vent sites from one another makes them ideal for the study of biogeography and natural history [37]. Our results suggest that *Thermococcus* diversity may be correlated in a preliminary manner with sample location and environmental traits, and suggests that they may be a broad biogeographical distribution pattern for these organisms both within and between thermal sites. The phenotypic diversity between groups is reflected in the IGS patterns, but only loosely observed in the highly conserved 16S rRNA gene sequences. Future research will attempt to expand on this study by analyzing the genetic and phenotypic traits of other *Thermococcus* spp. Hyperthermophilic members of the genera *Methanococcus* and *Archaeoglobus* are also ubiquitous in marine thermal habitats and may likewise demonstrate a pattern similar to that of *Thermococcus* spp.

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