Phenotypic Characterization, DNA Similarities, and Protein Profiles of Twenty Sulfur-Metabolizing Hyperthermophilic Anaerobic Archaea Isolated from Hydrothermal Vents in the Southwestern Pacific Ocean

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We performed phenotypic and physiological studies with 20 hyperthermophilic microorganisms isolated from hydrothermal vents located in the North Fiji Basin (southwestern Pacific Ocean) at a depth of 2,000 m. These isolates were strict anaerobes that were regular to irregular coccoids and used elemental sulfur in their metabolism. Growth was observed at temperatures ranging from 50 to 101°C. The DNA base compositions varied from 43 to 60 mol%. All of these organisms were heterotrophs and fermented peptides to acetate, isovalerate, isobutyrate, and propionate. They contained both diether and tetraether lipids in their membranes, which indicates that they belong to the domain *Archaea*. DNA-DNA hybridization experiments revealed that there were two distinct homology groups, which correlated well with results obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis of soluble whole-cell proteins, and these groups corresponded to the genera *Pyrococcus* and *Thermococcus*. Five isolates exhibited levels of DNA-DNA relatedness with *Pyrococcus abyssi* ranging from 71 to 100% and produced almost identical protein patterns. The remaining isolates formed a weakly homogeneous group based on DNA-DNA similarity data and protein patterns; the results of unweighted pair group cluster analyses suggested that these isolates were members of five new species of the genus *Thermococcus*.

Since the first discovery of deep-sea hydrothermal vents near the Galapagos Islands (12) in 1977, several new active vents in the Pacific and Atlantic Oceans have been explored (for a review see reference 42). Novel hyperthermophilic archaea (50) have been isolated from these extreme ecosystems and have been described. Some of these organisms have been identified as new species (13, 43).

The new isolates that have been described are autotrophs or heterotrophs, but most belong to the group that contains the neutrophilic sulfur metabolizers. This metabolic group includes six genera (the genera *Pyrococcus*, *Thermococcus*, *Hyperthermus*, *Pyrodicticum*, *Desulfurococcus*, and *Staphylothermus*) that were first described on the basis of shallow-water isolates (18, 19, 47, 53, 55, 56). Representatives of all of these genera except the genus *Hyperthermus* have also been found among the deep-sea isolates and assigned to new species (17, 25, 30, 41). However, several other fully described isolates have not been assigned to previously described or new species, probably because their morphological and physiological features did not distinguish them from each other or previously isolated species (26, 39, 40, 43, 44). All of these isolates are regular to irregular cocci that are motile or nonmotile. They are strict anaerobes and metabolize elemental sulfur, although some grow without sulfur and are not strictly sulfur dependent.

In this paper we report and discuss the results of a polyphasic characterization (which included determinations of morphology, physiology, protein profiles, G+C contents, and DNA-DNA hybridization data) and tentative identification of 20 anaerobic, chemoorganotrophic, hyperthermophilic, sulfur-

metabolizing strains that were isolated from the deep-sea hydrothermal vents discovered in 1989 in the southwestern Pacific Ocean.

MATERIALS AND METHODS

Isolates and reference strains. Samples of hydrothermal fluids, chimney, and invertebrates were collected by using the manned submersible vessel "Nautilus" during the cruises designated "Biolau" and "Starmer" organized in 1989 by the Institut Français de Recherche et Exploitation de la Mer (IFREMER) in the Lau Basin (site "Hine Hine"; 22°32'S, 176°43'W) and the North Fiji Basin (site "White Lady"; 16°59'S, 173°55'W), respectively. The maximum temperature of the fluids at the latter site was around 300°C, the pH was around 4 to 5, and the salinity was less than 0.3 mol/liter (1). The maximum temperature measured in the Lau Basin fluids was 400°C, the pH was around 2, and there was a high concentration of heavy metals (20).

A total of 20 hyperthermophilic strains were isolated from the North Fiji Basin samples (17). Another isolate was obtained from a sample prepared by scraping (under anaerobic conditions) the external surface of a gastropod (*Alviniconcha hessleri*) shell collected in the Lau Basin and transferred into and rinsed with degassed seawater. The resulting scrapings were inoculated into SME medium (47) and processed like the other samples. One of the North Fiji Basin isolates, strain GE5, has been described previously as the type strain of *Pyrococcus abyssi* (17) and was used in this study as a reference strain. The isolates and reference strains used in this study are listed in Table 1.

Pyrococcus furiosus DSM 3638, *Pyrococcus woesei* DSM 3773, *Thermococcus stetteri* DSM 5262, *Thermococcus litoralis* DSM 5474, and *Thermococcus celer* DSM 2476 were obtained from the Deutsche Sammlung von Mikroorganismen (DSMZ), Braunschweig-Stöckheim, Germany. *Pyrococcus abyssi* GE5^T (T = type strain) was obtained from our laboratory in Roscoff, France. *Pyrococcus* sp. strain GB-D was kindly provided by Carl O. Wirsén, Woods Hole Oceanographic Institution, Woods Hole, Mass., and *Pyrococcus* sp. strain AL-2 was kindly provided by Anna-Louise Reysenbach, University of Indiana, Bloomington.

Culture conditions. Isolates and reference strains were cultured in standard YPS medium (17). Briefly, 1 liter of YPS medium contained artificial seawater (23.4 g of NaCl, 10.8 g of MgCl₂ · 6H₂O, 4.0 g of Na₂SO₄, 0.7 g of KCl, 0.2 g of NaHCO₃, 0.09 g of KBr, 0.025 g of SrCl₂ · 6H₂O, 0.025 g of H₃BO₃, 0.003 g of NaF), 0.2 g of CaCl₂, 3.0 g of piperazine-*N,N'*-bis(ethanesulfonic acid) disodium salt (PIPES buffer; Sigma Chemical Co., St. Louis, Mo.), 4 g of Bacto Yeast Extract (Difco), and 0.4 g of Bacto Peptone (Difco). The pH was adjusted to 6.8, resazurin (1 mg liter⁻¹) was added, and the medium was autoclaved for 20 min.

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TABLE 1. Some characteristics of our isolates and other hyperthermophilic heterotrophs isolated from submarine hydrothermal vent environments

Species or strain(s)	Origin	Temp range (°C)	Substrate(s) ^a	Electron acceptor(s) ^b	Volatile fatty acids produced	% Ether lipids ^c	G+C content (mol%) ^d	Reference(s) ^e
<i>Thermococcales</i>								
<i>Pyrococcus</i> spp. (and <i>Pyrococcus</i> -like strains)								
<i>Pyrococcus furiosus</i>	Sediment of shallow marine solifatare, Vulcano, Italy, and MacDonald Seamount (-2,500 m)	70–103	Starch, maltose, cellobiose, pyruvate	NR, S ⁰ , cystine, polysulfides ^f	Acetate, isovalerate, isobutyrate, propionate	20	38.3–38.9 (37.1)	18, 24, 27
<i>Pyrococcus woesei</i>	Sediment of shallow marine solifatare, Vulcano, Italy	70–103	Starch, glycogen, maltose, cellobiose, pyruvate, H ₂	NR, S ⁰	NA ^g	Tr	(37.5)	54
GBD	Piece of chimney from Guaymas Basin (-2,000 m)	65–103	20-amino-acid mixture	NR, S ⁰ , cystine	NA	81	(39.5)	26
AL2	Piece of "flange" at Juan de Fuca Ridge (-2,200 m)	60–108	Casein	NR, S ⁰	NA	85	41.0	44
ES4	Piece of "flange" at Juan de Fuca Ridge (-2,200 m)	66–108	Starch, glycogen, cellobiose, 20-amino-acid mixture	S ⁰ , cystine	NA	72	47 (55.5) ^h	39, 40
KOD1	Sediment of shallow marine solifatare, Kagoshima, Japan	65–100	Casamino Acids	NR, S ⁰	NA	P ⁱ	38.0	37
<i>Pyrococcus abyssus</i> GE5 ^T	Hydrothermal fluid, North Fiji Basin (-2,000 m)	65–102	Casein, gelatin, 20-amino-acid mixture, pyruvate	NR, S ⁰ , cystine, polysulfides	Acetate, isovalerate, isobutyrate, propionate, heptanoate	85	44.3 (45.0)	17
GE1	Hydrothermal fluid, North Fiji Basin (-2,000 m)	65–95	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate, heptanoate	20	43.2	This study
GE7	Hydrothermal fluid, North Fiji Basin (-2,000 m)	65–98	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Isovalerate, isobutyrate, acetate, isocaproate, propionate	20	45.6	This study
GE9	Sulfide deposit, North Fini Basin (-2,000 m) ⁻¹	65–98	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	NA	80	44.0	This study
GE23	Chimney fragment, North Fiji Basin (-2,000 m)	65–100	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate	40	42.8	This study
GE27	Sulfide deposit, North Fiji Basin (-2,000 m)	65–100	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate, isocaproate	80	44.0	This study
<i>Thermococcus</i> spp. (and <i>Thermococcus</i> -like strains)								
<i>T. litoralis</i>	Sediment of shallow marine solifatare, Lucrino and Vulcano, Italy, and Java	55–98 and 50–96	Gelatin, casein, pyruvate, 20-amino-acid mixture	NR, S ⁰ , cystine	NA	NA	38.1–40.0	4, 38
<i>T. celer</i>	Sediment of shallow marine solifatare, Vulcano, and MacDonald Seamount	75–97	Gelatin, casein, 20-amino-acid mixture, sucrose	NR, S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate	Tr	56.9 (56.3)	53
<i>T. profundus</i>	Hydrothermal fluid, Mid-Okina Trough (-1,400 m)	50–90	Gelatin, casein	S ⁰	NA		(52.5)	31
<i>T. stetteri</i>	Sediment of shallow marine solifatare, Kuril Islands, Russia	55–95 and 75–98	Starch, pectin, gelatin, 20-amino-acid mixture	S ⁰ , cystine	Acetate, isovalerate, isobutyrate	NA	50.0–52.0	36
GE2	Hydrothermal fluid, North Fiji Basin (-2,000 m)	65–100	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate	60	55.0	This study
GE3	Rock debris, North Fiji Basin (-2,000 m)	65–90	20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, butyrate, isobutyrate, isocaproate, heptanoate, valerate	100	56.6	This study
GE6	Hydrothermal fluid, North Fiji Basin (-2,000 m)	50–95	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Isovalerate, isobutyrate, acetate, isocaproate, propionate, valerate	40	58.8	This study
GE8	Sulfide deposit, North Fiji Basin (-2,000 m)	60–98	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Isovalerate, isobutyrate, acetate, isocaproate, propionate, valerate	80	54.3 (53.7)	This study

GE18	Hydrothermal fluid, North Fiji Basin (-2,000 m)	60-95	Gelatin, 20-amino-acid mixture	S ⁰ , cystine	Acetate, isovalerate, isobutyrate	60	59.7	This study
GE19	Chimney fragment, North Fiji Basin (-2,000 m)	60-95	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, heptanoate, propionate, isocaproate	40	57.6	This study
GE20	Chimney fragment, North Fiji Basin (-2,000 m)	60-95	Gelatin, 20-amino-acid mixture	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate, isocaproate, butyrate	40	57.6	This study
GE21	Chimney fragment, North Fiji Basin (-2,000 m)	60-95	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, isocaproate	60	54.8	This study
GE22	Chimney fragment, North Fiji Basin (-2,000 m)	65-95	Gelatin, 20-amino-acid mixture	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate	40	51.7	This study
GE24	Rock debris, North Fiji Basin (-2,000 m)	65-95	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Isovalerate, acetate, isobutyrate, propionate, isocaproate	40	59.5	This study
GE25	Rock debris, North Fiji Basin (-2,000 m)	65-95	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate, isocaproate	40	57.4	This study
GE26	Sulfide deposits, North Fiji Basin (-2,000 m)	60-95	Gelatin, 20-amino-acid mixture	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate	40	56.2 (55.6)	This study
GE30	Shell of gastropod, Lau Basin (-2,200 m)	65-90	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Isovalerate, acetate, isobutyrate, propionate, isocaproate	NA	56.0	This study
GE31	Sediment (anhydrite), North Fiji Basin (-2,000 m)	60-95	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate, isocaproate	NA	55.0	This study
GE32	Sediment (anhydrite), North Fiji Basin (-2,000 m)	60-95	Gelatin, 20-amino-acid mixture, pyruvate	S ⁰ , cystine	Acetate, isovalerate, isobutyrate, propionate, isocaproate	NA	57.0	This study
ES1	Polychaete worm, chimney wall, Juan de Fuca Ridge (-2,200 m)	50-91		NR, S ⁰	NA	P	(58.6)	39
KS1, KS2, and KS8	Sediment of shallow marine solfatara, Kodakara Island, Japan	60-97	Casain, gelatin, 20-amino-acid mixture	S ⁰ (NR for KS8)	NA	P	(55.0, 53.2, 50.6)	22
<i>Desulfurococcus</i> <i>Staphylothermus marinus</i>	Sediment of shallow marine solfatara, Vulcano, Italy; piece of chimney, East Pacific Rise, 11°N site (-2,500 m)	65-98		S ⁰	Acetate, isovalerate	NA	34.9 (35.3)	19
<i>Desulfurococcus</i> sp. strains S and SY	Piece of chimney, East Pacific Rise, 11°N site (-2,500 m)	50-90 and 50-95	Casain (SY), 20-amino-acid mixture	NR, S ⁰	NA	68 (S), 78 (SY)	(52.0, S; 52.4, SY)	25
<i>Pyrodicticum abyssi</i>	Sediment and pieces of chimney, Guamas Basin (-2,000 m); sediment and hot fluids, Kolbeinsey Ridge, Iceland (-103 m)	80-110	Casain, gelatin, Casamino Acids, starch, glycogen, lactose, raffinose, formate, acetate, H ₂	NR, S ⁰ , S ₂ O ₃ ²⁻	Acetate, isovalerate, isobutyrate, butanol	>50	59.3-60.0 (59.0-61.9)	41, 47
<i>Hyperthermus butylicus</i>	Sediment of shallow marine solfatara, Sao Miguel, Azores	80-108	H ₂	NR, S ⁰	Acetate, butanol, propionate, phenylacetate	85	(55.6)	52, 55

^a All of the organisms grow on complex proteinaceous compounds, including yeast extract, peptone, tryptone, casein hydrolysate, and beef extract. Although H₂ produced during fermentation generally inhibits growth, it stimulates growth in some organisms.

^b As far as we know, all of the organisms grow by fermentation (and not by anaerobic sulfur respiration), and S⁰ or other sulfur compounds are used as electron sinks (46).

^c The values are the percentages of tetraether lipids.

^d Unless indicated otherwise, the G+C contents were determined by the melting point method. The values in parentheses were determined by a direct HPLC analysis (see Materials and Methods).

^e Including publications in which organisms were first described and selected publications in which aspects of metabolism are discussed.

^f NR, not required.

^g NA, not available.

^h Determined by H. Klump as cited by Di Ruggiero et al. (15).

ⁱ P, ether lipids are present.

The following solutions, which had been sterilized by filtration, were then added: 10 ml of a trace element solution (3), 1 ml of a 10 mM Na_2WO_4 solution, 1 ml of a 25 mM FeCl_3 solution, 10 ml of a 7% (wt/vol) KH_2PO_4 solution, and 10 ml of a 14% (wt/vol) NH_4Cl solution. The medium was then completed by adding 0.5% (wt/vol) sulfur that had been sterilized by steaming at 100°C for 1 h on 3 successive days. Anaerobiosis was achieved by applying a vacuum to the medium and saturating it with N_2 (2). Finally, the medium was reduced by adding a sterile solution of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (final concentration, 0.025% [wt/vol]). Cell biomass samples for protein, DNA, and lipid analyses were obtained from late-log-phase cultures grown at 85°C in 500-ml serum bottles containing 200 ml of medium and incubated in a rotary shaker water bath (200 rpm; Bioblock, Illkirch, France) filled with dimethyl silicon oil (Prolabo, Paris, France). Plates containing YPS medium solidified with Gelrite (Sigma) (16) were incubated for 3 to 7 days at 85°C in anaerobic jars (Don Whitley Scientific, Ltd., Shipley, Yorkshire, England) containing an $\text{N}_2\text{-H}_2\text{-CO}_2$ (90:5:5) gas phase (100 kPa).

Nutritional requirements and temperature range. The experiments to determine nutritional requirements and temperature ranges were performed in 27-ml Hungate culture tubes (Bellco, Vineland, N.J.) containing 10 ml of medium and N_2 (100 kPa) in the headspace unless indicated otherwise. Temperature ranges for growth were determined by incubating culture tubes in thermoregulated heating modules (Bioblock) whose wells were filled with dimethyl silicon oil in order to facilitate heat transfer.

The ability of the isolates to use single carbon sources for growth was determined on YPS medium without yeast extract and peptone. This mineral medium was supplemented with each carbon source (starch, gelatin, maltose, yeast extract, peptone, tryptone, pyruvate, casein hydrolysate, and beef extract) at a final concentration of 0.2% (wt/vol) and a filter-sterilized solution of vitamins (10 ml/liter) (3). A mixture of 20 amino acids, each at a concentration of 0.1 mM, was also tested. Each tube was inoculated with 100 μl of an exponentially growing culture (final concentration, ca. 10^8 cells per ml) and incubated at 85°C. All tests were performed in duplicate, and a control in which no carbon substrate was added was used as a baseline since some growth due to nutrients added with the inoculum was expected.

The possible electron acceptors L-(–)-cystine, SO_3^{2-} , SO_4^{2-} , $\text{S}_2\text{O}_3^{2-}$, and Fe_3^+ were each tested at a final concentration of 30 mM in YPS medium without sulfur and Na_2SO_4 under the conditions described above for carbon sources; 1 mM Ti(III) citrate was used as the reducing agent in place of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ in these experiments. A control in which no electron acceptor was added was used as a baseline since growth due to fermentation was expected. Sulfur was added along with each electron acceptor as a control for possible growth inhibition by the substrates. *Pyrococcus abyssi*, *Pyrococcus furiosus*, *T. litoralis*, and *T. stetteri* were used as reference organisms in the growth tests.

Growth was monitored by measuring H_2S production as described by Cord-Ruwisch (11). The final cell density, which was reached after 1 to 7 days of incubation (depending on the isolate), was estimated by direct cell counting (see below).

Light microscopy. Cells were observed with an Olympus model BH2 light microscope equipped with a phase-contrast oil immersion objective (magnification, $\times 100$) and were counted by using a Petroff-Hausser chamber (depth, 0.02 mm). To determine motility, glass slides were heated to 95°C immediately before observation.

Analysis of metabolic products. Volatile fatty acids were analyzed by using acidified cultures samples (final HCl concentration, 1 M) and a model 5300 gas chromatograph (Carlo Erba, Rodano, Italy) equipped with a flame ionization detector and a Carbowax 20 M column (Supelco, Inc., Bellefonte, Pa.); the air pressure was 120 kPa, the carrier gas was H_2 (40 kPa), the combustion gas was H_2 (80 kPa), the oven temperature was programmed to increase from 80 to 200°C at a rate of 3°C/min, the detector temperature was 120°C, and the injector temperature was 150°C. The limit of detection for the volatile fatty acids produced in the culture medium was around 0.05 mM.

Exoprotease activity. Exoprotease activity was tested at 85°C by using azocasein as the substrate. After 1 ml of a culture in the late log phase was centrifuged for 5 min at $17,700 \times g$, 0.1 ml of the supernatant was mixed with 0.5 ml of a 2% (wt/vol) azocasein (Sigma) solution and 0.4 ml of 0.1 M phosphate buffer (NaH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; Sigma), pH 7.2. After 60 min the reaction was stopped by adding 1 ml of 10% (wt/vol) trichloroacetic acid, and the solution was incubated at room temperature for 30 min. The solution was then centrifuged for 5 min at $19,000 \times g$, and 1 ml of the supernatant was mixed with 1 ml of 1 N NaOH. The optical density at 450 nm of each solution was determined with a Uvikon 930 spectrophotometer. Uninoculated medium was used as the control.

Lipid analysis. Lyophilized cell material was extracted by the method of Bligh and Dyer (5) as modified by White et al. (49). The lipids were analyzed by the method of Ross et al. (45).

Cellular protein extraction and PAGE. Cell pellets were washed twice with artificial seawater, suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM dithiothreitol, 1 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), 1 mM EDTA, and 0.4 mM phenylmethylsulfonyl fluoride, and disrupted by sonication in the presence of glass beads (diameter, 1 mm). After centrifugation at $19,000 \times g$ and 4°C for 20 min, the supernatant was centrifuged twice for 40 min at $19,000 \times g$ and 4°C before we performed a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis as described by Laemmli (31). Equal amounts of protein (protein concen-

trations were estimated by the Bradford method [8] by using a Bio-Rad assay kit [Bio-Rad Laboratories, Richmond, Calif.] from all samples were loaded onto 10% polyacrylamide gels (5% stacking gels) along with the following molecular weight standards: rabbit muscle phosphorylase β (molecular weight, 97,400), bovine serum albumin (66,200), hen egg white ovalbumin (42,699), bovine carbonic anhydrase (32,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). The gels were stained with Coomassie brilliant blue R250.

DNA isolation and base composition. DNA was extracted as described by Charbonnier et al. (10) with the following modifications. After phenol-chloroform extraction, the DNA was precipitated by adding 0.8 volume of isopropanol, spooled onto a glass rod, washed twice with cold 70% (vol/vol) ethanol, and allowed to air dry. The DNA recovered was then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and treated with 50 μg of RNase A (Sigma) per ml for 2 h at 37°C, and this was followed by phenol-chloroform extraction, spooling, and dissolution in TE buffer as described above. The DNA preparations from all strains were dialyzed together overnight at 4°C against two changes of 2 liters of TE buffer. The purity of each DNA preparation was such that the A_{260}/A_{280} and A_{260}/A_{230} ratios were consistently 1.8 to 1.9 and 2.0 to 2.3, respectively. The DNA preparations were electrophoresed on 0.8% agarose minigels to test for impurities and degradation and always formed well-defined bands at approximately 22 to 25 kb.

The G+C content of the DNA of each isolate was determined from its melting point in TE buffer as described by Mandel and Marmur (33) and Marmur and Doty (34). A calibration curve was obtained by using *Clostridium perfringens* DNA (31.0 mol% G+C), *Escherichia coli* DNA (51.3 mol% G+C), and *Micrococcus lysodeikticus* DNA (72.0 mol% G+C) (all obtained from Sigma) as standards. A direct nucleotide analysis of isolates GE8, GE26, GE30, and GE31 was also performed by high-performance liquid chromatography (HPLC) after the DNAs were digested with nuclease P1 as described by Zillig et al. (57) and Mesbah et al. (35); calf thymus DNA (42 mol% G+C; Sigma) and phage λ DNA (48.9 mol% G+C; Boehringer Biochemica, Mannheim, Germany) were used as the standards in this analysis.

DNA-DNA hybridization. Levels of genetic relatedness were determined by performing DNA-DNA slot blot hybridization experiments (6, 29). Duplicate aliquots containing 100 and 200 ng of each genomic DNA were denatured by boiling them for 10 min in 0.4 M NaOH and were transferred onto positively charged membranes (Zeta-Probe; Bio-Rad) by using a slot blot apparatus (Bio-Rad). Each membrane contained DNA from the bacterium *Alteromonas carraghenovora* (45 mol% G+C; kindly provided by Tristan Barbeyron, Roscoff, France) as a negative control. Prehybridization for 2 h and hybridization were performed at the optimal temperature (28) in buffer containing $4 \times \text{SSC}$ ($1 \times$ is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% blocking reagent (Amersham International, Les Ulis, France), 5% dextran sulfate, 100 μg of denatured and sheared salmon sperm DNA per ml, and 20% deionized formamide. Probes (DNAs that were sheared by sonication to lengths of 500 to 2,000 bp and were heat denatured) were labelled by using an ECL random prime labelling kit (Amersham International) and were added (10 ng/ml) to the prehybridized membranes in hybridization buffer, and the preparations were incubated overnight (16 h). Hybridized membranes were washed twice at 65°C (15 min each) successively in $1 \times \text{SSC}$ –0.1% SDS, $0.5 \times \text{SSC}$ –0.1% SDS, and $0.1 \times \text{SSC}$ –0.1% SDS. Chemiluminescence was detected with an ECL detection kit as recommended by the manufacturer (Amersham). The membranes were exposed to Hyperfilm (Amersham) for 10 to 20 min at room temperature. Separate tests involving self-hybridization of strain GE5^T DNA revealed that there was a linear response between the amount of target DNA and the signal intensity under the exposure conditions used (data not shown). As the labelling efficiency varied a little from one probe DNA to another, the exposure time was adjusted to ensure that the intensities of the signals for 100 and 200 ng remained within the linear part of the response curve. The intensities of the signals were measured with a densitometer (Vernon, Paris, France). To reprobe the blots, the probes were removed from the membranes by using the following protocol: after washing for 5 min in $5 \times \text{SSC}$ at room temperature, the membranes were stripped of the probe by incubating them for 2 min in 0.1% SDS at 95°C. We found that the same membrane could be stripped and reprobed at least five times with no detectable loss of sensitivity. The signal (maximum peak area) produced by self-hybridization of the probe with homologous target DNA was defined as 100%, and the final percentage of similarity for each pair of strains was the average of the values calculated for the duplicate slots containing 100 and 200 ng of target DNA. The complete similarity matrix (20 by 20) was converted to a distance matrix with the transformed elements equal to $1 - (\text{percentage of similarity}/100)$ and then used for a cluster analysis by the unweighted pair group method of analysis program from the PHYLIP package (version 3.4) included in the GCG software package version 8.0 (Genetic Computer Group, University of Wisconsin Biotechnology, Madison, Wis.).

RESULTS

Morphology. The cells of all of the isolates were regular to irregular cocci that were 0.8 to 2 μm in diameter. Motility was observed with seven isolates (GE1, GE3, GE7, GE23, GE19, GE20, and GE27). All strains grew on Gelrite plates at 85°C

TABLE 2. Nutritional requirements of the isolates

Strain or species	Growth in the presence of the following substrate ^a :								
	Yeast extract	Peptone	Tryptone	Beef extract	Casein hydrolysate	Gelatin	20-amino-acid mixture	Pyruvate	Starch
GE1	+++	+++	+++	++	++++	+++	++	+	-
GE2	+++	+++	+++	++	++++	++++	++	+	-
GE3	++++	-	+	++	+++	(+)	(+)	+	-
GE5 ^T	+++	+++	++++	+++	++++	+++	++	+	-
GE6	+++	+++	+++	++	++++	+++	+	+	-
GE7	++++	+++	++++	++	++++	+++	++	+	-
GE8	+++	+++	++++	++	++++	+++	+	+	-
GE9	++++	+++	+++	++	++++	+++	++	+	-
GE18	++++	++	+	++	++++	+	++	-	-
GE19	+++	++	+	-	++++	(+)	+	+	-
GE20	++++	++	(+)	(+)	++	(+)	+	-	-
GE21	+++	+++	+	++	++++	+	++	+	-
GE22	+++	+++	+++	++	++++	++++	(+)	-	-
GE23	+++	+++	++++	+++	++++	++	++	+	-
GE24	++++	+++	-	++	++++	(+)	+++	+	-
GE25	+++	+++	+	++	+++	(+)	(+)	+	-
GE26	+++	+++	+	++	++++	++	++	-	-
GE27	+++	+++	++	++	++++	(+)	+	+	-
GE30	+++	++	+	++	++	+	(+)	+	-
GE31	++++	+++	+	++	++++	+	++	+	-
GE32	+++	+++	++	+++	++++	+	++	+	-
<i>Pyrococcus furiosus</i>	+++	+++	+++	+++	+++	+++	++	+	++
<i>T. litoralis</i>	+++	++	+	++	+++	+++	++	+	++
<i>T. stetteri</i>	++++	+++	+	+++	++++	+++	++	-	++

^a Growth was measured by determining the final cell density and was expressed as follows: -, no growth; (+), 5×10^6 to 10^7 cells per ml; +, 10^7 to 5×10^7 cells per ml; ++, 5×10^7 to 10^8 cells per ml; +++, 10^8 to 2×10^8 cells per ml; +++++, $>2 \times 10^8$ cells per ml. The concentration of each proteinaceous substrate tested was 0.2% (wt/vol), and pyruvate, maltose, and starch were each added at a concentration of 0.5% (wt/vol). The concentration of each of the amino acids in the 20-amino-acid mixture was 0.1 mM. All of the isolates and the reference strains grew very well (+++ to +++) in YPS medium; none of them except *Pyrococcus furiosus* grew on maltose.

and had similar colonial morphologies; the colonies were white or cream, convex, and 0.5 to 2.0 mm in diameter.

Nutritional requirements. The isolates did not grow autolithotrophically in mineral medium containing elemental sulfur and CO₂ but grew well heterotrophically and anaerobically in the presence of S⁰. The nutritional requirements of the isolates were very similar. Most of the isolates grew very well on complex proteinaceous substrates (yeast extract, peptone, tryptone, beef extract, and casein hydrolysate) and proteins (gelatin); however, there were a few exceptions (Table 2). For instance, isolate GE3 did not grow on peptone and grew poorly on gelatin; isolate GE19 did not grow on beef extract and grew poorly on gelatin; isolate GE20 grew poorly on tryptone, beef extract, and gelatin; and isolate GE24 did not grow on tryptone and grew poorly on gelatin. All of the isolates were able to use a mixture of the 20 natural amino acids as a sole carbon and energy source, although the growth was generally weaker than the growth observed with complex substrates, especially for strains GE3, GE22, GE25, and GE30. No growth was observed on carbohydrates (starch and maltose). Most of the isolates grew poorly on pyruvate; the exceptions were isolates GE18, GE20, GE22, and GE26, which did not grow at all on this substrate.

None of the isolates was able to use any of the electron acceptors tested except S⁰ and L-(-)-cystine. Therefore, they appeared to be sulfur dependent.

Temperature ranges. The maximum growth temperatures (Table 1) for all isolates were between 90 and 101°C. The most thermophilic isolates grew at temperatures ranging from 60 to 98°C (GE8) or from 65 to 101°C (GE2, GE7, GE9, GE23, and GE27). The less thermophilic isolates (GE3 and GE30) grew at temperatures between 65 and 90°C. All of the other isolates grew at temperatures up to 95°C, and the minimum growth temperature was 65°C (GE1, GE22, GE24, and GE25), 60°C (GE18, GE19, GE20, GE21, GE26, GE31, and GE32), or 50°C (GE6).

Fermentation products. When grown on YPS medium, all of the isolates produced acetate, isovalerate, isobutyrate, and propionate (Table 1). About 3 to 8 μmol of acetate and isovalerate per ml, 1 to 2 μmol of isobutyrate per ml, and up to 0.2 μmol of propionate per ml were produced in 20 h. The relative proportions of these products depended on the isolate. Butyrate was produced only by GE3 and GE20 (about 2 and 0.5 $\mu\text{mol ml}^{-1}$), and caproate was detected only in GE3. Other volatile fatty acids were also detected in some isolates but usually at very low concentrations ($<0.1 \mu\text{mol ml}^{-1}$). In all of the isolates, growth was accompanied by exponential H₂S production which paralleled growth. The H₂S concentration reached up to 13 $\mu\text{mol ml}^{-1}$ when cells entered the stationary phase (data not shown).

Exoprotease activity. Exoprotease activity was detected in three isolates, GE8, GE19, and GE22. GE8 and GE19 exhib-

FIG. 1. Electrophoretic analysis of cellular protein extracts of the isolates and four reference strains. Samples were electrophoresed on a 10% polyacrylamide gel that was stained with Coomassie brilliant blue. Lane a contained molecular weight markers, whose sizes (in kilodaltons) are indicated on the left. Clusters A, B, B1, and B2 are the electrophoresis protein profile groups described in the text.

ited levels of proteinase activity similar to the levels observed in *T. stetteri*, but the level of activity in GE22 was almost twofold higher.

Lipid composition. Archaeal ether lipids were present in all of the isolates tested. No significant amounts of (bacterial) methyl esters were detected. Diether and tetraether forms were found in all of the isolates except GE3, which contained only traces of diether lipids (Table 1).

Cellular protein profiles. Different cellular protein patterns were observed (Fig. 1), and these patterns allowed us to divide the isolates into two main clusters. Cluster A strains were characterized by four bands at molecular weights of 98,000, 90,000, 55,000, and 48,000, which were absent in cluster B strains. Cluster B strains produced three characteristic protein bands at molecular weights of 97,000, 93,000, and 50,000.

Five isolates (GE1, GE7, GE9, GE23, and GE27) belonged to cluster A and produced profiles that were almost identical to the *Pyrococcus abyssi* GE5^T profile and rather similar to the *Pyrococcus furiosus* profile. Identical profiles were produced by isolates GE7, GE9, and GE23 and by isolates GE5 and GE27, while GE1 produced a slightly different profile than GE27. The other isolates were classified in cluster B, which appeared to be less homogeneous than cluster A. However, two subclusters were distinguished; one of these subclusters included isolates GE19, GE20, GE21, and GE30 (subcluster B1), and the other included isolates GE2, GE6, GE18, GE24, GE25, GE26, GE31, and GE32 (subcluster B2). Three strains (GE3, GE8, and GE22) did not group with either of the subclusters. The profiles of the subcluster B1 strains were quite similar to the *T. stetteri* profile, although some bands of the subcluster B1 strains appeared to be less intense. The *T. celer* protein pattern was quite similar to the isolate GE3 pattern.

DNA base composition. The G+C contents of the isolates varied from 43.2 to 59.7 mol% (Table 1). Three G+C content groups were distinguished; one group contained isolates which had G+C contents ranging from 43 to 46 mol% (GE1, GE5^T, GE7, GE9, GE23, and GE27), the second group contained isolates which had G+C contents ranging from 51 to 54 mol% (GE8 and GE22), and the third group contained isolates which had G+C contents ranging from 55 to 60 mol% (the rest of the isolates).

Levels of DNA similarity. The results of DNA-DNA hybridization experiments performed with our isolates and seven reference strains are shown in Table 3. The hybridization experiments revealed that there were two major DNA similarity groups. One group contained isolates GE1, GE7, GE9, GE23, and GE27, which exhibited levels of similarity of at least 68% and hybridized strongly (65 to 100%) with GE5, the type strain of *Pyrococcus abyssi*. The levels of DNA similarity with the other *Pyrococcus* strains were never more than 42% (GE27 and GBD), and the levels of similarity with the three *Thermococcus* species were much lower (the maximum level of similarity was 21% for GE27 and *T. litoralis*). This group appeared to be weakly connected to the rest of the isolates (average level of similarity, 6%).

The levels of DNA similarity between pairs of the other isolates fluctuated between 0 and 100%, but most pairs (12 of 15) exhibited levels of DNA similarity between 60 and 100%. Isolates GE2, GE6, GE18, GE19, GE20, GE21, GE24, GE25, GE26, GE30, GE31, and GE32 did not exhibit levels of similarity of more than 58% with the *Pyrococcus* reference strains (GE19 and GBD) or more than 42% with the *Thermococcus* reference strains (GE20 and *T. stetteri*). Isolates GE3, GE8, and GE22 did not exhibit levels of similarity of more than 28%,

TABLE 3. Levels of DNA-DNA relatedness among isolates and reference strains

Source of unlabelled DNA	% Relatedness to labelled DNA from ^a .																			
	GE1	GE2	GE3	GE5 ^T	GE8	GE9	GE19	GE20	GE22	GE23	GE25	GE30	GE32	<i>Pyrococcus furosus</i>	<i>Pyrococcus woesei</i>	GBD	AL2	<i>T. celer</i>	<i>T. stetteri</i>	<i>T. lionialis</i>
GE1	100	2	0	65	8	74	1	6	8	83	0	5	5	17	18	23	11	11	9	8
GE2	2	100	18	12	22	10	58	50	28	2	40	65	38	3	8	34	10	25	30	6
GE3	0	30	100	0	21	3	40	16	19	1	30	18	15	0	0	2	0	10	0	0
GE5 ^T (<i>Pyrococcus abyssii</i>)	76	10	0	100	8	96	1	7	7	98	5	5	9	24	25	41	31	9	9	20
GE8	2	35	16	5	100	7	40	4	35	4	28	14	4	0	0	1	0	3	28	0
GE9	70	5	0	84	0	100	0	4	100	100	0	8	0	19	21	28	21	0	8	2
GE19	0	74	58	0	54	10	100	35	28	1	76	93	96	2	11	58	6	32	18	0
GE20	10	58	10	6	10	2	45	100	11	0	28	24	10	0	0	0	0	17	42	0
GE22	0	6	10	0	28	0	16	8	100	5	21	20	0	0	0	0	2	2	37	7
GE23	68	5	0	79	10	98	2	6	2	100	2	0	5	12	7	14	17	3	0	7
GE25	5	41	18	7	23	2	39	30	29	0	100	68	58	1	4	6	1	9	20	4
GE30	8	60	17	2	18	8	84	32	30	0	70	100	87	4	4	38	2	28	13	2
GE32	0	25	9	15	4	0	25	15	16	0	28	52	100	0	0	4	0	5	14	0
<i>Pyrococcus furiosus</i>	21	3	0	15	0	7	0	0	0	10	0	0	0	100	78	2	0	13	3	12
<i>Pyrococcus woesei</i>	19	3	0	13	0	12	0	0	0	11	0	20	0	85	100	2	1	9	2	12
GBD	22	24	0	38	0	13	30	0	0	19	12	2	0	8	7	100	56	0	3	5
AL-2	9	10	0	41	0	15	3	0	0	23	5	24	8	10	11	62	100	5	7	7
<i>T. celer</i>	8	18	18	4	8	0	29	15	0	4	10	20	0	9	3	0	0	8	9	4
<i>T. stetteri</i>	9	34	10	6	41	12	35	25	40	7	44	0	20	0	4	1	2	4	100	9
<i>T. lionialis</i>	11	5	0	9	2	4	0	3	9	9	0	0	0	16	15	0	0	8	12	100
GE6	5	65	19	0	48	8	78	96	46	0	100	0	88	ND ^b	6	40	2	26	ND	ND
GE7	74	8	0	85	8	89	1	15	5	98	2	0	7	ND	13	32	27	5	0	8
GE18	0	65	28	0	52	8	86	98	63	0	70	2	90	2	9	39	3	22	15	0
GE21	8	71	8	0	36	4	70	96	41	1	75	ND	48	0	1	8	2	15	ND	ND
GE24	2	39	6	0	0	1	57	57	ND	0	75	ND	61	0	0	15	1	14	ND	ND
GE26	3	60	26	0	ND	10	65	96	ND	5	90	88	71	ND	1	19	0	18	ND	21
GE27	73	18	1	100	0	98	27	39	ND	85	10	15	18	28	27	42	35	11	8	ND
GE31	5	71	16	0	34	7	87	100	41	3	97	98	97	3	5	43	2	22	ND	ND

^a The level of hybridization is the average level of binding for at least four replicates (see Materials and Methods). Only the values corresponding to the complete matrix (20 by 20) were used to construct an unweighted pair group dendrogram (Fig. 2).

^b ND, not determined.

FIG. 2. Unweighted pair group dendrogram based on DNA-DNA hybridization data. We used 12 isolates and 8 reference strains for which a complete distance matrix (20 by 20) was obtained. A distance value of 20 on the dendrogram is equivalent to a level of hybridization homology of 60%. The G+C content ranges of the strains are represented by the boxes on the right.

and the highest levels of similarity with reference strains were 38% (GE8 and *T. stetteri*) and 41% (GE22 and *T. stetteri*).

A complete matrix (20 by 20) was derived from the DNA-DNA hybridization data and was used to construct a dendrogram (Fig. 2). Two main clusters were observed. One cluster contained *Pyrococcus abyssi* GE5^T, GE1, GE9, and GE23 (GE7 and GE27 were not included in the tree construction analysis), the three *Pyrococcus* species (*Pyrococcus furiosus*, *Pyrococcus woesei*, and strain GBD), and strain AL2. The type strain of *T. litoralis* joined this cluster at a low level of similarity. The second cluster contained the other strains; two of these strains (GE22 and GE8) grouped with *T. stetteri*. *T. celer* appeared to differ from our isolates and joined the second cluster at a low level of similarity.

DISCUSSION

All of the isolates were coccoid, sulfur-metabolizing heterotrophs and grew at high temperatures under strictly anaerobic conditions. These characteristics corresponded to characteristics described previously for hyperthermophilic heterotrophic

Archaea. The presence of ether lipids in the membranes of our isolates allowed us to assign them to the domain Archaea (50).

The previously described heterotrophic, anaerobic, marine, hyperthermophilic archaea are represented by the genera *Thermococcus* (53), *Pyrococcus* (18), *Desulfurococcus* (25), *Staphylothermus* (19), *Pyrodictium* (47), and *Hyperthermus* (55). Our isolates share several morphological features with certain strains belonging to these genera, particularly the genera belonging to the *Thermococcales* (the genera *Thermococcus* and *Pyrococcus*). For instance, constricted duplex forms, which are characteristic of members of the *Thermococcales* (51), were observed during exponential growth of our isolates. Although this form of division can also be found in *Hyperthermus* strains, it is considerably less frequent in this genus than in the *Thermococcales*. When our strains were grown in nonagitated cultures, aggregates of cells were frequently observed. However, these clusters differed from those of *Staphylothermus* (19) or *Hyperthermus* strains (52), which form clumps containing up to 100 cells. A doubling time of less than 2 h, as observed for our isolates, is a common characteristic of members of the *Thermococcales* (51). Our isolates produced the same volatile fatty acids that are found in members of the genera *Thermococcus*, *Pyrococcus*, *Staphylothermus* (7), and *Desulfurococcus* (25), but did not produce any butanol as has been observed for *Hyperthermus* strains (52) and *Pyrodictium abyssi* (41). Other phenotypic characteristics, such as motility, growth temperature range, nutritional requirements, and sulfur dependence, appeared to be too variable in the members of the six genera mentioned above to be useful for classification purposes.

It has been reported previously that *T. celer* (14), *Pyrococcus woesei*, and *Thermococcus* sp. strain AN1 (32) contain only diether in their membranes, whereas *Desulfurococcus* strains contain tetraether as a major component (14). In *The Prokaryotes*, 2nd ed., it was proposed that the presence of the diether lipids is a signature for the *Thermococcales* (51). However, *Pyrococcus abyssi* GE5^T, which was clearly assigned to the *Thermococcales* (17), contains only low amounts of diether. Many of our strains had both di- and tetraethers in their membrane lipids, but the tetraethers were dominant in most of the strains. Similar lipid compositions were also found for recently described isolates assigned to the order *Thermococcales* (22, 37) and to the new species *Thermococcus profundus* (30). These observations indicate that lipid composition (ratio of diether to tetraether) is not a good criterion for classification of thermophilic, sulfur-metabolizing archaea.

The G+C contents of our isolates ranged from 43% to 60 mol% and could be divided into three groups (43 to 46, 50 to 54, and 55 to 60 mol%). The first G+C content group corresponded to the genus *Pyrococcus*, whose members have G+C contents ranging from 38 to 45% mol%. The members of this group were the most thermophilic organisms and had the shortest doubling times, features which are typical of *Pyrococcus* species. These findings correlated well with the classification based on protein profiles (Fig. 1) and were further confirmed by DNA-DNA hybridization data (Table 3); the protein profiles of the *Pyrococcus* isolates were included with the group A protein profiles at a level similar to the level predicted by hybridization data. This is consistent with the findings of Cato et al. (9), who reported that strains with levels of DNA similarity greater than 80% usually produce identical protein patterns. The members of the second G+C content group (50 to 54 mol%) could belong to the genus *Desulfurococcus* or the genus *Thermococcus*, whose members have G+C contents ranging from 41 to 52 and 38 to 57 mol%, respectively. It is more likely that the members of the second G+C content group belong to the genus *Thermococcus* than that they belong

to the genus *Desulfurococcus* because isolates GE8 and GE22 exhibited levels of similarity of around 40% with *T. stetteri*, had similar protein profiles, and exhibited strong exoprotease activity. The G+C contents of the members of the third G+C content group (54 to 60 mol%) are similar to the G+C content of *Thermococcus* strains, and these organisms have the characteristics described for this genus, but they exhibit only low levels of similarity (<30%) with reference species.

In this study, we found that slot blot hybridization in which a nonisotopic method is used can provide reliable information about the levels of DNA-DNA relatedness of a large number of isolates as long as optimum conditions are used (see above). However, the results obtained from slot blot hybridization experiments should be considered to be only semiquantitative because of the strong influence of experimental factors (6). Nevertheless, the results obtained by this method were in good agreement with the results that we obtained previously for a smaller set of strains by using a method (S1 nuclease and radioactively labelled DNA) (15a, 17) which is generally considered the most accurate method available (21).

The dendrogram in Fig. 2 is a convenient way to represent the levels of DNA relatedness among the isolates, but for the reasons cited above it should not be overinterpreted as providing quantitative evidence concerning the genetic distances between isolates. Nevertheless, some general conclusions concerning the genetic relationships among the isolates can be drawn. The level of DNA-DNA similarity for species boundaries was set arbitrarily at 60% by Johnson (27). More recently (48), a value of 70% was proposed, and this value seems to be better suited as a species boundary value for procaryotes. Even when the lower value (which equals a distance of 20 on the dendrogram) was used, several of our isolates represented new species. Our data suggest that GE8 and GE22 could represent new *Thermococcus* species that are closely related to *T. stetteri* and that the remaining isolates could be placed in three other new *Thermococcus* species, one containing isolates GE19, GE32, GE30, and GE25), one containing isolates GE20 and GE2, and one containing isolate GE3. This conclusion seems to be particularly true for isolate GE3, which appears to be only distantly related to all of the other strains. It is surprising that *T. litoralis* appeared to be rather close to the genus *Pyrococcus* on the dendrogram. Indeed, the G+C content of *T. litoralis* (39.1 mol%) is within the range of G+C contents for the genus *Pyrococcus*; reclassification has been suggested previously (51).

Assignment of our isolates to the *Thermococcales* is not surprising since the enrichment procedures which we used were those described for the *Thermococcales*. In addition, members of the *Thermococcales* are widely distributed in the hydrothermal systems of the planet (43). However, it is surprising how diverse the isolates belonging to the *Thermococcus* group were compared with the isolates belonging to the *Pyrococcus* group, which were quite homogeneous. Our tentative identification of five novel *Thermococcus* species for 15 isolates that seemed to be almost identical after phenotypic and physiological studies clearly shows the need for genomic studies of the heterotrophic sulfur-metabolizing coccoid hyperthermophiles.

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