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Investigation of structure and antigenic capacities of *Thermococcales* cell envelopes and reclassification of "*Caldococcus litoralis*" Z-1301 as *Thermococcus litoralis* Z-1301

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Abstract Fourteen strains of hyperthermophilic organotrophic anaerobic marine Archaea were isolated from shallow water and deep-sea hot vents, and four of them were characterized. These isolates, eight previously published strains, and six type strains of species of the order *Thermococcales* were selected for the study of cell wall components by means of thin sectioning or freeze-etching electron microscopy. The cell envelopes of most isolates were shown to consist of regularly arrayed surface protein layers, either single or double, with hexagonal lattice (p6) symmetry, as the exclusive constituents outside the cytoplasmic membrane. The S-layers studied differed in center-to-center spacing and molecular mass of the constituent protein subunits. Polyclonal antisera raised against the cells of 10 species were found to be species-specific and allowed 12 new isolates from shallow water hot vents to be identified as representatives of the species *Thermococcus litoralis*, *Thermococcus stetteri*, *Thermococcus chitonophagus*, and *Thermococcus pacificus*. Of the 7 deep-sea isolates, only 1 was identified as a *T. litoralis* strain. Thus, hyperthermophilic marine organotrophic isolates obtained from deep-sea hot vents showed greater diversity with regard to their S-layer proteins than shallow water isolates.

Key words Submarine hot vents · Hyperthermophilic Archaea · S-layers · Immunochemical identification

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Introduction

Organotrophic marine Archaea have been actively studied over the last 15 years and represent now the most numerous group of hyperthermophiles. They are represented by five genera belonging to three orders: *Thermococcus* (Zillig et al. 1983) and *Pyrococcus* (Fiala and Stetter 1986) of the order *Thermococcales*; *Staphylothermus* (Fiala et al. 1986), belonging to *Desulfurococcales*; and *Pyrodictium* (Stetter et al. 1983) and *Hyperthermus* (Zillig et al. 1991), which are members of *Pyrodictiales* (Stetter 1996). Among these groups of organisms, the order *Thermococcales* is the most widely represented one, including a total of 18 species. Despite taxonomic diversity, all these organisms are phenotypically very similar. Their cells are irregular cocci, in most cases motile by means of a bundle of flagella. The physiology of hyperthermophilic organotrophic marine Archaea is also quite similar (Schönheit and Schafer 1995): they are obligate anaerobes, organotrophs fermenting complex organic substrates, amino acids, or pyruvate, and, being sensitive to hydrogen formed in the course of fermentation, require elemental sulfur as an external electron acceptor when grown in closed vessels. All representatives are extreme thermophiles or hyperthermophiles with growth optimum at 85°–88°C (*Thermococcus* and *Staphylothermus*), or 100°–103°C (*Pyrococcus*, *Pyrodictium*, and *Hyperthermus*). In an attempt to find phenotypic criteria for species differentiation within this group of Archaea, cell protein profiles were suggested as the most reliable feature (Marteinsson et

al. 1995). The objectives of this work were to compare the S-layer proteins as exclusive wall components of different representatives of hyperthermophilic marine organotrophic Archaea and to elaborate a species-specific identification method based on antibody typing.

Materials and methods

Sources of isolation

Samples from Guaymas were kindly provided by V.F. Galchenko (Institute of Microbiology, Russian Academy of Sciences, Moscow) and were obtained from a depth of 2000 m by means of a *Pisces* submersible apparatus during the voyage of the *D. Keldysh* research vessel. Samples from shallow water hot vents of Matupi Harbor, New Guinea, and the Bay of Plenty, New Zealand, were obtained during the 18th voyage of the *A. Nesmeyanov* scientific vessel. The description of sampling sites and sampling procedures were reported elsewhere (Namsaraev et al. 1994). Samples of water and sand from Kuril Islands were kindly provided by V.G. Tarasov (Institute of Marine Biology, Vladivostok, Russia).

Strains

Type strains of *Pyrococcus* or *Thermococcus* species used in this study were either maintained in the collections of authors or obtained from DSMZ: *Pyrococcus furiosus* DSM 3638^T, *Pyrococcus abyssi* GE5, *Thermococcus celer* DSM 2476^T, *Thermococcus litoralis* DSM 5474^T, *Thermococcus stetteri* K3 (DSM 5262^T), *Thermococcus profundus* DT 5432^T, *Thermococcus peptonophilus* JCM 9653^T, *Thermococcus chitonophagus* DSM 10152^T, *Thermococcus gorgonarius* W12 (DSM10395^T), and *Thermococcus pacificus* P4 (DSM 10394^T). The two other identified strains also used in this work were *Pyrococcus abyssi* GE23 (Marteinsson et al. 1995) and *Thermococcus stetteri* K15 (Miroshnichenko et al. 1989). We also used some unidentified or not validated strains previously published: "*Caldococcus litoralis*" (Svetlichny et al. 1987) and isolates GE3, GE6, GE20, GE21, and GE25 (Marteinsson et al. 1995).

Isolation and cultivation

Isolation of pure cultures of hyperthermophilic organotrophic marine Archaea was done either by transfer of separate colonies from the surface of Gelrite (Sigma) -solidified medium (Miroshnichenko et al. 1998) or by serial dilutions in a liquid medium of the same composition. Hyperthermophilic organotrophic marine isolates were cultured in 15-ml Hungate tubes on the same medium. The physiology of new isolates, the G + C content of the DNA, and the DNA–DNA homology were studied as described elsewhere (Miroshnichenko et al. 1998).

Electron microscopy studies

Thin sections were prepared as described earlier (Miroshnichenko et al. 1994). For the freeze-etching, the cells were frozen in Freon and specimens were prepared as described by Sleytr et al. (1988), or they were frozen in liquid propane and cleaving and platinum carbon shadowing were done as described by Borovjagin et al. (1987). Samples were etched for 2–4 min at −100°C. Preparations were examined in a JEM-100C (Jeol) electron microscope.

Characterization of cell envelope proteins

Cells of strains studied were centrifuged for 20 min at 10000 *g*. Pellets were resuspended in distilled water and sonicated (22 kHz, 2 min). Then lysates were centrifuged for 30 min at 15000 *g*. Pellets were resuspended in 8 M urea. SDS-PAGE was performed according to Laemmli (1970). The following molecular weight standards (Biolabs, Bevezly, MA, USA) were used: 212000, 158000, 116000, 97000, 66000, 55000, 42000, and 36000.

Immunological methods

For the immunization experiments, cells of the eight type strains, *Thermococcus stetteri* K15, and "*Caldococcus litoralis*," were grown in 15-ml Hungate tubes, centrifuged, and then washed twice with 0.15 M NaCl. The density of the final suspensions was not less than 10^9 cells ml^{-1}. Polyclonal antisera were raised in rabbits by injection of 1 ml of culture suspensions: hypodermic injection: after 7 days intramuscular injection; after 7 days intravenous injection. Seven days after the last immunization, about 20 ml of blood was taken from an ear vein. Blood cells were pelleted by centrifugation (5000 *g*, 20 min) and supernatants were used for immunological experiments. Immunodiffusion was performed according to Ouchterlony (1953). For immunoblotting, proteins from SDS gels were transferred for 2–2.5 h at 300 mA in a transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) to nitrocellulose membranes (0.45 μm; Biorad). After transfer, membranes were incubated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Tween 20 (TBST) containing 1% blot qualified bovine serum albumin (BSA), at 4°C overnight. Membranes were incubated with the diluted antisera for 1 h, washed, incubated with alkaline phosphatase conjugated second antibodies, and visualized by using color-developing substrates, nitro blue tetrazolin and 5-bromo-4-chloro-3-indolyl-phosphate, according to manufacturer's (Promega, Madison, WI, USA) instructions.

Results

Isolation and characterization of new isolates

Fourteen strains of hyperthermophilic organotrophic marine Archaea were obtained, which showed anaerobic

growth on the peptone-containing marine medium supplemented with elemental sulfur at pH 6.5 and 85°C (Table 1). Four new isolates have been characterized (Table 2). Cells of all strains were irregular cocci, 1–2 μm in diameter, with flagella; the cell envelopes consisting of one or two S-layers (Fig. 1). The cell wall of isolate Z-1519 consisted of two S-layers (Fig. 1b). This double S-layer appearance is not a preparation artifact, as was recently confirmed in an independent study of another strain of *Thermococcus* (Marteinsson et al., unpublished observation). The new hyperthermophilic organotrophic marine isolates differed in their relation to elemental sulfur; its presence in the medium was obligately required for the growth of isolates P2-3 and 1519 and only stimulating for isolates Z-1614 and MW. The G + C content of the DNA varied from 35.4 to 57.1 mol%.

Table 1. Isolation of hyperthermophilic marine organotrophic Archaea from deep-sea and shallow water habitats

Site	Location	Designation of isolates
Guaymas	Deep sea	Z-1614, Z-1519
Matupi Harbor	Shallow water	MW
Bay of Plenty	Shallow water	P2-3
Kuril Islands	Shallow water	K1A, K1B, E1, E3, E4, E6, Sh1AM, Sh1B, 2104, 2705

S-layer structure and composition of the new isolates and type strains

The S-layers of 18 strains of hyperthermophilic marine organotrophic strains were studied. In most freeze-etching preparations, a hexagonal symmetry of the S-layer lattice was observed (Fig. 2a–c). In three preparations, however, the hexagonal lattice was not visible. Thus, the S-layers could be characterized only as possessing a periodic structure (Fig. 2d). The S-layers of the investigated archaeal strains showed different center-to-center spacings, ranging from 11.5 to 20.9 nm (Table 3).

The molecular mass of major cell envelope proteins was determined for several strains. One or two major proteins were found to be present in cell envelopes of archaeal strains studied; their molecular mass varied from 60 to 230 kDa (Table 3).

Table 2. Characteristics of several new deep-sea and shallow water isolates

Strain	Morphology	T°_{opt}	pH_{opt}	Requirement for S°	G + C content of DNA, mol%
Z-1614	Irregular cocci with flagella, cell envelope with one S-layer	86	6.8	S	35.4
Z-1519	Irregular cocci with flagella, cell envelope with two S-layers of different size	90	7.0	R	57.1
P2-3	Irregular cocci with flagella, cell envelope with one S-layer	82	6.7	R	51.9
MW	Irregular cocci with flagella, cell envelope with one S-layer			S	42.5

S, stimulating; R, required

Table 3. S-layer characteristics of the investigated strains

Strain	Lattice type	Center-to-center spacing (nm)	Molecular mass of major protein(s) (kDa)
Pyrococcus furiosus DSM 3868	Hexagonal	14.6	170; 75
P. abyssi GE5	Hexagonal	14.4	ND
P. abyssi GE23	Hexagonal	15.9	ND
Thermococcus stetteri K3	Hexagonal	14.5	230; 80
T. stetteri K15	Hexagonal	18.3	210; 80
T. celer DSM 2746	Hexagonal	20.9	180
T. gorgonarius W12	Hexagonal	19.5	70
T. pacificus P4	Hexagonal	19.1	55
"Caldococcus litoralis"	?	ND	120; 60
GE3	Hexagonal	14.8	ND
GE6	Hexagonal	17.1	ND
GE20	Hexagonal	16.6	ND
GE21	Hexagonal	11.5	ND
GE25	Hexagonal	17.7	ND
P2-3	Hexagonal	18.6	60
Z-1519	Hexagonal	18.4	125; 60
Z-1614	?	ND	135
MW	?	ND	ND

ND, not determined; ?, type of lattice could not be determined

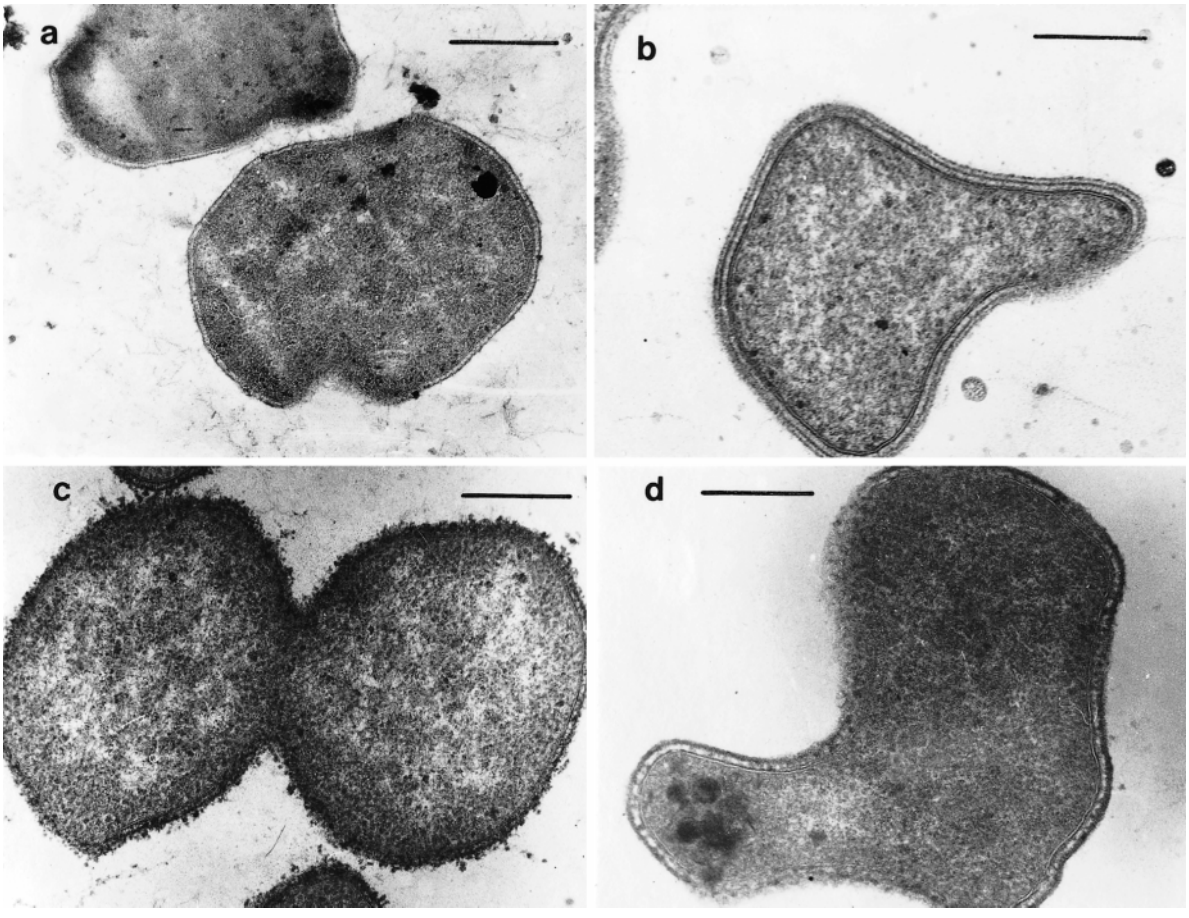

Fig. 1a–d. Electron photomicrographs of thin-sectioned cells of the new hyperthermophilic organotrophic marine isolates: Z-1614 (**a**), Z-1519 (**b**), P2-3 (**c**), and MW (**d**). *Bar* 500 nm

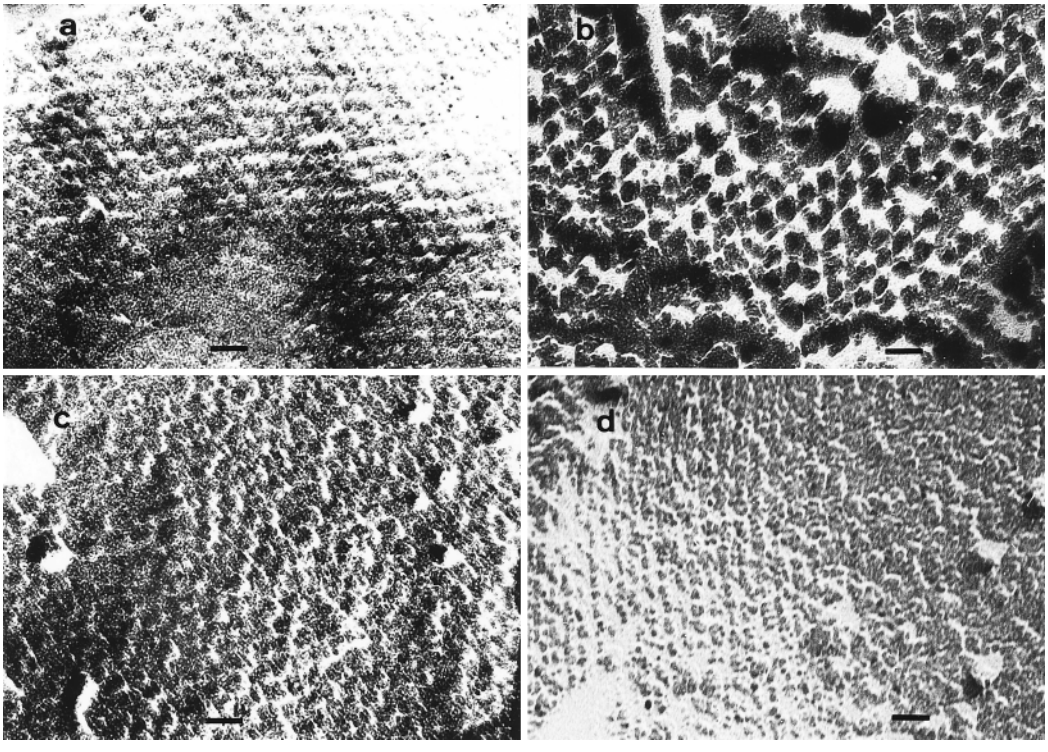

Fig. 2a–d. Electron photomicrographs of freeze-etched cells of hyperthermophilic organotrophic marine Archaea: *Pyrococcus furiosus* (**a**), *Thermococcus celer* (**b**), Z-1519 (**c**), and "*Caldococcus litoralis*" (**d**). *Bar* 32 nm

Table 4. Results of immunoblotting reaction of antisera with cells of the new isolates

Strain	Antiserum to								
	P. furiosus	T. celer	T. stetteri K3	"C. litoralis"	T. chitonophagus	T. peptonophilus	T. profundus	T. gorgonarius	T. pacificus
GE3	−	−	−	−	−	−	−	−	−
GE6	−	−	−	−	−	−	−	−	−
GE20	−	±	±	±	±	−	±	±	±
GE21	−	−	−	−	−	−	−	−	−
GE25	−	−	−	−	−	−	−	−	−
Z-1614	−	−	−	+	−	−	−	−	−
Z-1519	−	−	−	−	−	−	−	−	−
P2-3	−	−	−	−	−	−	−	−	+
MW	−	−	−	+	−	−	−	−	−
K1A	−	−	+	−	−	−	±	−	−
K1B	−	−	+	−	±	−	−	−	±
E1	−	−	−	±	+	−	−	−	−
E3	−	−	+	−	−	−	−	−	−
E4	−	−	−	−	+	−	−	−	−
E6	−	−	+	−	−	−	−	−	−
Sh1AM	−	−	−	+	±	−	−	−	−
Sh1B	−	−	−	+	±	−	−	−	−
2104	−	−	+	−	−	−	−	−	−
2705	−	−	±	−	+	−	−	−	−

+, strong reaction; ±, weak reaction; −, no reaction

Immunochemical analyses

Antisera to whole cells of ten archaeal strains were raised, including the eight type strains of *P. furiosus*, *T. celer*, *T. stetteri*, *T. profundus*, *T. peptonophilus*, *T. chitonophagus*, *T. gorgonarius*, and *T. pacificus*; and two other strains, *T. stetteri* K15 and "*Caldococcus litoralis*." Quality of antisera obtained was checked by the Ouchterlony method, and antisera specificity was tested by immunoblotting.

It was found, in immunoblotting analysis, that antisera diluted 1:100 or 1:1000 cross-reacted with cells of all type strains tested (data not shown). When diluted 1:5000, antisera reacted strongly only with the strains against which they had been raised (Fig. 3). Antisera to *T. stetteri* strain K3 and strain K15 cross-reacted with cells of both strains, thus confirming that this method was species- rather than strain-specific. Antiserum to "*Caldococcus litoralis*" reacted with cells of *T. litoralis*^{T} DSM 5474, indicating that "*Caldococcus litoralis*" was a strain of *T. litoralis*.

Cells of all new and unidentified isolates were used in the immunoblotting analyses with nine antisera (Table 4). Thirteen isolates gave positive reaction with four antisera: isolates Z-1614, MW, Sh1AM, and Sh1B, with antiserum to *T. litoralis*; E1, E4, and 2705, with that to *T. chitonophagus*; isolates K1A, K1B, as well as strains E3, E6, and 2104, were identified as strains of *T. stetteri* and isolate P2-3 as *T. pacificus*. Some of these results were tested by parallel DNA–DNA hybridization analyses. A high level of DNA–DNA homology between the evaluated strains and the type strains confirmed the results of immunochemical analyses (Table 5). Six deep-sea isolates gave no positive reaction with any of the antisera used.

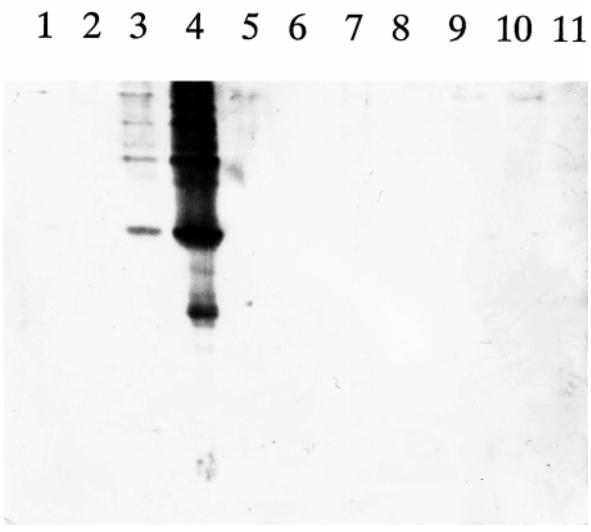

Fig. 3. Western blot analysis of different strains with antiserum against *P. furiosus* showing strong reaction with *P. furiosus* cell proteins (*lane 4*), weak reaction with *T. celer* cell proteins (*lane 3*), and no reaction with cell proteins of Z-1614 (*lane 1*), "*C. litoralis*" (*lane 2*), K1B (*lane 5*), K1A (*lane 6*), E1 (*lane 7*), E3 (*lane 8*), E6 (*lane 9*), 2104 (*lane 10*), and 2705 (*lane 11*). Antiserum was diluted 1:5000

Discussion

Crystalline protein layers on the surface of prokaryotic cells have been termed surface layers (S-layers), and have been found in many bacteria belonging to all phylogenetic branches and virtually all archaeal species studied (for recent compilation, see Messner and Sleytr 1992; Sleytr et al. 1996). In many archaeal species, the S-layers constitute the

Table 5. The DNA–DNA cross-hybridization reaction between the new and reference strains of the genus *Thermococcus*

Strain	% of DNA–DNA cross-hybridization with	
	T. litoralis DSM 5474	T. steteri K3
"*C. litoralis*"	96	ND
Z-1614	71	ND
MW	69	ND
K1B	ND	97
T. litoralis	100	ND
T. stetteri	ND	100

ND, not determined

only cell wall structure, which is attached to the cytoplasmic membrane (Sleytr et al. 1988; König 1988; Phipps et al. 1991; Baumeister and Lembcke 1992). Most S-layers possess hexagonal (p3 or p6) lattice symmetry. Up to now, oblique and square (p2 or p4) lattice symmetry was shown only for several hyperthermophilic Archaea such as representatives of the *Crenarchaeota*, *Desulfurococcus mobilis* (Baumeister et al. 1990) and *Pyrolobus fumarii* (Blöchl et al. 1997), and *Ferroglobus placidus* (Hafenbradl et al. 1996), belonging to the *Euryarchaeota*.

This study was aimed at the characterization of the cell walls of hyperthermophilic Archaea of the genera *Pyrococcus* and *Thermococcus*, representing the order *Thermococcales* of the kingdom *Euryarchaeota*. All isolates of this group described so far possess cell walls consisting of one or two S-layers outside the cytoplasmic membrane. The double S-layer was studied in detail (by freeze-etching and isolation of sheets) only for *Pyrobaculum organotrophum* (Phipps et al. 1991). However, numerous thin sections revealed that the double S-layer could be recognized as a common feature of *Thermococcales*. This structure was first shown for *Pyrococcus woesii* (Zillig et al. 1987) but was found later to be fairly widespread among representatives of *Thermococcus* and *Pyrococcus* genera: *T. stetteri* (Miroshnichenko et al. 1989), *T. celer* (Baumeister et al. 1990), *Pyrococcus abyssi* (Erauso et al. 1993), *T. chitonophagus* (Huber et al. 1995), *T. peptonophilus* (Gonzales et al. 1995), *T. fumicolans* (Godfroy et al. 1997), and *T. pacificus* (Miroshnichenko et al. 1989). The cell walls of the four new isolates described here also possess either one or two S-layers. Isolates Z-1614 and MW, identified as members of *T. litoralis*, are covered with a single S-layer. We did not observe double S-layers on thin sections of strain P2-3, which was identified as *T. pacificus*. Although reported for the type strain of this species, the S-layer of this organism was described as very fragile and easily destructed (Miroshnichenko et al. 1989). This fact might explain its absence on thin sections of strain P2-3.

With the exception of three strains, all other strains examined in this study were shown to have S-layers with hexagonal lattice symmetry. These three isolates belong to *T. litoralis*, and their cell wall structures need further characterization. S-layers of all the other strains differed in the center-to-center spacing and molecular mass of major proteins (see Table 3). In several cases, the values obtained for strains belonging to the same species were fairly close, but in other cases these differed significantly (*T. stetteri* K3 and K15, GE20, and GE21). Although in Bacteria the structure and even the occurrence of an S-layer are strain-specific features, in Archaea the available data might be exploited for differentiating species. Their presence could be associated with a very important common role of S-layers in archaeal cells, where they often occur as the only cell wall component. For example, in *Thermoproteus tenax* (Messner et al. 1986; Wildhaber and Baumeister 1987), *Pyrobaculum organotrophum* (Phipps et al. 1991), and *Methanocorpusculum sinense* (Pum et al. 1991) it was demonstrated that S-layers are involved in cell shape determination and cell division. However, more evidence is needed to prove the species-specificity of cell wall components of *Thermococcales*.

Immunochemical experiments indicated that the antigenic capacity of *Thermococcales* cell envelopes might be used for species identification. Immunoblotting analyses with highly diluted antisera provided reliable differentiation of species belonging to the genus *Thermococcus* and allowed identification of several new and previously published strains. Three isolates identified as the strains of *T. litoralis* originated from shallow water hot vents of Kurils and New Guinea and the fourth was isolated from deep-sea hot vents of Guaymas. The new strains of *T. stetteri* and *T. pacificus* originated from the same sites as the type strains of these species (Kurils and New Zealand, respectively). By contrast, *T. chitonophagus*, originating from Guaymas deep-sea vents, was now found only among shallow water isolates. Thus, all 12 shallow water isolates tested could be identified, whereas six of seven deep-sea strains failed to react with any of the nine species-specific antisera used. These strains either are representatives of the recently described species of *Thermococcales* that are not yet included in our antisera bank or belong to new taxa. Our findings confirm the considerable taxonomic diversity among deep-sea microbial communities in comparison to shallow water populations.

"*Caldococcus litoralis*" (Svetlichny et al. 1987) was previously published as a representative of a novel genus, based on a significant 14% difference in the G + C content with the only representative of *Thermococcus*—*T. celer*—known at that time. Later a new species, *Thermococcus litoralis*, was published (Neuner et al. 1990). Immunoblotting analyses reported here indicated that "*C. litoralis*" and the type species of *T. litoralis* represent the same species. This evidence is supported by the results of the DNA–DNA hybridization (96% homology between two strains). Thus, we propose to reclassify "*Caldococcus litoralis*" as *Thermococcus litoralis* Z-1301.

Phenotypically, strain Z-1301 is very close to the type strain of *Thermococcus litoralis*, as it has the same pH and temperature range of growth, ferments peptides, and does not obligately require elemental sulfur for growth. The only significant difference is the presence of flagella in strain Z-1301, whereas the type strain of *T. litoralis* is not flagellated. However, the presence of flagella is a strain-specific feature, as was shown for *T. stetteri* (Miroshnichenko et al. 1989). There is also a 3% difference in G + C content of the DNA of both strains. We include these strain-specific differences in the following emended description of *Thermococcus litoralis.*

Emended description of *Thermococcus litoralis*

Thermococcus litoralis sp. nov., Neuner, Jannasch, Belkin and Stetter. Cells are regular or irregular cocci 0.5–3.0 μm, not flagellated, or with a tuft of flagella. Obligate organtrophs, utilizing peptides. The presence of elemental sulfur in the medium stimulates growth but is not obligately required. Hyperthermophiles, growing between 55° and 100°C with an optimum at 85°–88°C. pH for growth ranges from 4.0 and 8.0 with optimum at 6.0–6.4. Growth occurs at NaCl concentration from 1.8% to 6.5%, with the optimum at 2.5%. G + C content of DNA, 38–41 mol%. Inhabits shallow and deep-sea submarine hot vents. Type strain: *Thermococcus litoralis* NS-C (DSM 5473).

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