Growth Physiology of the Hyperthermophilic Archaeon *Thermococcus litoralis*: Development of a Sulfur-Free Defined Medium, Characterization of an Exopolysaccharide, and Evidence of Biofilm Formation

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Nutritional characteristics of the hyperthermophilic archaeon *Thermococcus litoralis* have been investigated with emphasis on the development of a sulfur-free, defined growth medium, analysis of an exocellular polysaccharide, and formation of a biofilm. An artificial-seawater-based medium, containing 16 amino acids, adenine, uracil, vitamins, and trace elements, allowed *T. litoralis* to attain growth rates and cell densities similar to those found with complex media. Four amino acids (alanine, asparagine, glutamine, and glutamate) were not included due to their lack of effect on growth rates and cell yields. In this medium, cultures reached densities of 10^8 cells per ml, with doubling times of 55 min (without maltose) or 43 min (with maltose). Neither the addition of elemental sulfur nor the presence of H_2 significantly affected cell growth. A sparingly soluble exopolysaccharide was produced by *T. litoralis* grown in either defined or complex media. Analysis of the acid-hydrolyzed exopolysaccharide yielded mannose as the only monosaccharidic constituent. This exopolysaccharide is apparently involved in the formation of a biofilm on polycarbonate filters and glass slides, which is inhabited by high levels of *T. litoralis*. Biofilm formation by hyperthermophilic microorganisms in geothermal environments has not been examined to any extent, but further work in this area may provide information related to the interactions among high-temperature organisms.

Hyperthermophiles—microorganisms growing optimally above 80°C and capable of growth above 90°C—have been isolated from several geothermal environments, including terrestrial hot springs and hydrothermal marine environments (64). Many such organisms have been isolated in pure culture; however, recent examination of 16S rRNA signatures of hot spring samples suggests only a small fraction of hyperthermophiles have been identified (5). To some extent, this can be attributed to an inability to effectively represent the ecological environment of individual hyperthermophiles under laboratory conditions. Consequently, only the most nutritionally versatile and ecologically competitive organisms are likely to be successfully isolated. To expand the quantity and diversity of isolated hyperthermophilic microorganisms for further scientific study or technological evaluation, better isolation and cultivation techniques need to be developed.

To date, isolation media used for most hyperthermophiles typically contain sources of peptides and oligosaccharides (9, 32). Although there is evidence that individual amino acids are taken in (67) and assimilated (10) by a number of hyperthermophiles, complex media are usually chosen due to the apparent inability of these organisms to utilize mixtures of single amino acids as sole carbon and/or energy sources (6). Many thermophilic archaea, such as *Thermococcus peptonophilus* (21), *Thermoplasma acidophilum* (60), *Hyperthermus butylicus* (72), and *Desulfurococcus* spp. (29), are reported to have a growth requirement for peptides. This may arise from an inability of the organisms to transport certain amino acids into

the cell, competition between the uptakes of several essential amino acids (51), or the greater thermal instability of single amino acids compared to their stabilization in peptides (68).

Defined media for studying the growth physiology of hyperthermophilic microorganisms in which suitable growth rates and yields can be obtained are generally not available. However, efforts to develop defined media for members of the *Thermococcales* have been reported (23, 24, 32, 52, 61, 69). *Pyrococcus furiosus* has been grown to 1.5×10^7 cells per ml in a partially defined medium containing tryptone (0.01 g/liter), trace elements, and 19 amino acids (minus tyrosine) (9). Cell densities were at least 10 times higher, however, for growth on peptide-based media or when the defined media were supplemented with pyruvate or saccharides (9, 33). Although evidence for direct monosaccharide utilization was not previously found for *P. furiosus* (19), Usenko et al. (67) have shown that this organism can take up glucose, which is metabolized intracellularly (34, 57). The low levels of direct glucose utilization in hyperthermophiles probably relate to rapid degradation at high temperatures, especially at 90°C and higher (34).

Although sulfur reduction is a common feature among hyperthermophiles (10, 33, 36, 37, 46), a few, such as *P. furiosus* (optimum temperature, 98 to 100°C) (19) and *Thermococcus litoralis* (optimum temperature, 88°C) (7, 48), can grow in its absence by producing high levels of H_2 , presumably as an alternative means of processing excess reducing equivalents (32). H_2 is required for some hyperthermophiles (32); however, since H_2 is inhibitory to many others, its production leads to weaker growth than that obtained in the presence of sulfur (27, 41, 52). Sulfur reduction apparently offsets this inhibition by forming H_2S instead of H_2 (32).

Biopolymer production, which may provide an alternative sink for excess reducing power, has been investigated in some

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less thermophilic archaea (38, 49, 63). The extreme thermoacidophile *Sulfolobus acidocaldarius* was observed to produce a sulfated, glucose-, mannose-, and galactose-containing exopolysaccharide during growth under optimal conditions (49). The mesophilic halophile *Haloferax mediterranei* was also found to synthesize a sulfated, exocellular polysaccharide as a function of growth (4), while *Methanosarcina thermophila*, a moderately thermophilic, acetogenic methanogen, produces a heteropolysaccharide outer layer (63) that may stabilize the cells at low osmotic pressures or aid in colonization (56). Yet to be investigated are whether exopolysaccharide (EPS) production is widespread among hyperthermophiles and how it is involved in biofilm formation (13, 17, 20).

Described here are efforts focusing on the nutritional requirements for the hyperthermophilic archaeon *Thermococcus litoralis* and leading to development of a sulfur-free defined medium for this organism. Arising from this work is evidence of a sulfated, mannan-like EPS, which seems to be involved in the formation of a biofilm. This finding supports the existence of attached hyperthermophilic consortia in geothermal environments, possibly containing yet-to-be-discovered microorganisms difficult to cultivate with current methodologies.

MATERIALS AND METHODS

Cultivation and cell enumeration procedures. *T. litoralis* (DSM 5473) and *P. furiosus* (DSM 3638) were cultivated in sealed 150-ml serum bottles containing 100 and 50 ml of culture medium, respectively. The bottles were heated to 98°C for 30 min, sparged with nitrogen, reduced with sodium sulfide (2 ml of 50-g/liter $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ per liter of culture), inoculated with late-exponential-phase cells to an initial density of 5×10^6 cells per ml, and sealed. Rezaurin (1 mg/liter) was used as a redox indicator. Cultivation proceeded without agitation at 88°C (for *T. litoralis*) and 98°C (for *P. furiosus*). Long-term storage of *T. litoralis* and *P. furiosus* was routinely accomplished by growing 5 ml of the organism in a 20-ml Hungate tube to approximately 10^8 cells per ml, adding 5 ml of 20% anaerobic glycerol, and storing at -70 or -20°C . Cultures were transferred by thawing at room temperature and inoculating 10% (vol/vol) into new media.

Cell densities were determined by epifluorescence microscopy with acridine orange (Sigma Chemical Co., St. Louis, Mo.) (25). Samples (1 ml) containing cells were added to 100 μl of 2.5% glutaraldehyde (Sigma Chemical Co.), vortexed, and fixed for approximately 5 min. An appropriate dilution of the sample and 200 μl of 1-g/liter acridine orange were added to sterile water to yield a final volume of 5 ml. After 2 min, the solution was pulled onto a prewetted, 25-mm-diameter, 0.2- μm -pore-size polycarbonate filter (Poretics, Livermore, Calif.) by using a 10-ml vacuum tower (Fisher Scientific, Pittsburgh, Pa.). Filters were placed on a glass slide with a drop of oil, covered with a coverslip, and viewed with a 100 $\times$ oil immersion objective on a Nikon HB-10101AF epifluorescent microscope. Ten grids (containing approximately 20 to 30 cells each) were counted for each filter and averaged for cell density calculations. Error for this procedure was determined by counting six independent samples from each of six bottles grown in the same manner and with the same inoculum. An average $\pm 9\%$ standard deviation was determined for a 95% confidence interval.

Growth physiology experiments. Nutritional requirements were investigated by utilizing an artificial-seawater-based medium consisting of (per liter) 25 g of NaCl, 2 g of MES [2-(*N*-morpholino)-ethanesulfonic acid], 1 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 g of Na_2SO_4 , 75 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 350 mg of KCl, 50 mg of NaBr, 20 mg of H_3BO_3 , 20 mg of KI, 10 mg of $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, 3 mg of $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 140 mg of K_2HPO_4 , and 100 mg of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$. Trace element solution (added at 10 ml/liter) contained (per liter) 150 mg of nitrilotriacetate, 50 mg of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 140 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 36.2 mg of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 1 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. A vitamin solution was also added (at 10 ml/liter) which contained (per liter) 2 mg of folic acid, 10 mg of pyridoxine-HCl, 5 mg of thiamine-HCl, 4 mg of riboflavin, 5 mg of nicotinic acid, 2 mg of biotin, 5 mg of DL-Ca-pantothenate, 0.1 mg of vitamin B₁₂, 5 mg of *p*-aminobenzoic acid, and 5 mg of DL-6,8-thioctic acid (lipoic acid). *P. furiosus* cultures were supplemented with 1 g of yeast extract, 5 g of tryptone, 2 g of maltose, and 10 g of S⁰ per liter. *T. litoralis* was initially grown with 1 g of yeast extract, and 5 g of tryptone per liter. All chemicals were obtained from Sigma Chemical Co. except for glycine, threonine, and tyrosine, which were obtained from Fisher Scientific. Liquid stock solutions were made of each base medium component, as well as the vitamin, trace elements, maltose, yeast extract, and tryptone solutions. Amino acids were added separately in powdered form. The solution was heated to dissolve the amino acids, adjusted to pH 6.0 with NaOH, and filter sterilized. Concentrated medium (1.2 $\times$) was used to reduce dilution errors for the addition of extra medium components, such as maltose, yeast extract, or tryptone, to batch cultures in comparison studies.

Artificial seawater was used as the base medium for the amino acid elimination

study with the following exceptions: the NaCl concentration was 15 g/liter, and MES was not included. Amino acids (19 at 0.1 g/liter), with the one under investigation being excluded, were added to the base medium with 2 g of maltose and 0.05 g of yeast extract per liter. Nucleotide bases were evaluated by adding adenine hemisulfate, cytosine, guanine, thymine, and uracil at a level of 0.01 g/liter.

The defined medium developed for *T. litoralis*, Rinker's defined medium (RDM), consisted of the basic salts, vitamins, and trace elements described above with the addition of 2 g of MES, 0.2 g of each of 16 amino acids (Arg, Asp, Cys, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val), and 0.01 g each of adenine and uracil per liter. The effects of NH_4Cl and selenium were evaluated by addition of 1 g/liter and 0.5 mg/liter, respectively, to RDM. The pH of the NH_4Cl culture (6.6) did not vary significantly from that of the control (6.7) at room temperature. The nutrient effect study was performed with a modified form of RDM with 0.05 g of each of 16 amino acids per liter and one of the following individual nutrients: 5 g of glucose, mannose, xylose, cellobiose, arabinose, galactose, starch, cellulose, isoleucine, glycine, or valine per liter, 5% (vol/vol) glycerol, or 1 g of pyruvate per liter. Each component was tested in a separate batch culture which was grown for 24 h. When maltose or yeast extract was included in subsequent experiments, 2 g/liter and 1 g/liter were added, respectively. For cultures grown on sulfur, a colloidal sulfur suspension (approximately 0.85 g of S⁰ per liter) was utilized (58). Hydrogen inhibition was evaluated by sparging bottles with 100% H₂ (high purity; National Welders, Charlotte, N.C.).

Biofilm formation. RDM was the base medium for investigating biofilm formation. The effects of maltose, yeast extract, and sulfur were investigated by adding 5, 1, and 10 g/liter, respectively, to the medium in 500-ml bottles containing 0.2- μm -pore-size polycarbonate filters or glass slides (Fisher Scientific). Cells were grown under quiescent conditions for 26 h (past stationary phase in all cases). For examination by epifluorescent microscopy, filters were removed, rinsed twice with sterile medium, and fixed in 2.5% glutaraldehyde (Sigma Chemical Co.) for at least 5 min. The filters were then stained in 0.04 g of acridine orange per liter in water for 2 min. Excess moisture was removed by briefly placing the filters under a vacuum. The filters were placed on glass slides and viewed with a 100 $\times$ oil immersion lens under a Nikon epifluorescence microscope as described above. Glass slides were removed from batch cultures, rinsed with sterile medium twice, and prepared for light microscopy with Congo red to stain the EPS and carbol fuchsin to distinguish the cells (1). Slides with biofilms were prepared for analysis by rinsing three times in sterile medium and then hydrolyzing the material with 5 ml of 3 N HCl at 98°C for 18 h. The composition of polymeric material on glass slides was determined by high-performance liquid chromatography (HPLC) on the hydrolysate (pH 7 achieved by addition of NaOH) using a Waters (Milford, Mass.) high-performance carboxylate column (4.6 by 250 mm) with a corresponding guard column at 30°C, a 1.0-ml/min flow of acetonitrile-water (75:25), and a Shimadzu refractive index detector (RID-6A). Polysaccharide in biofilms grown with the addition of maltose, yeast extract, and/or sulfur was measured by using the orcinol-sulfuric acid total carbohydrate assay (71a).

Electron microscopy. Biofilm samples for scanning electron microscopy were obtained as described above. Filters were immediately removed from the stationary-phase culture, rinsed twice in sterile medium, and fixed in Trump's fixative (44a) (pH 7.26). Suspension samples were obtained from stationary-phase cultures (1 ml), pulled down onto a polycarbonate filter, and fixed in 3% glutaraldehyde. The samples were dehydrated by washing with successively higher concentrations of ethanol, critical point dried in CO₂, and then sputter coated with gold. Viewing was performed with a JEOL JSM-35CF microscope.

Polysaccharide characterization. *T. litoralis* was obtained from (i) batch cultures grown on RDM supplemented with 2 g of maltose and 1 g of yeast extract per liter, (ii) continuous culture on RDM and 2 g of maltose per liter at a dilution rate of 0.2 liter/h, and (iii) continuous culture on RDM and 0.25 g of maltose per liter at a dilution rate of 0.6 liter/h. *P. furiosus* was cultivated as previously stated. EPS was purified by first separating the supernatant from the cells by centrifugation at $10,000 \times g$ for 30 min at 4°C. The resulting cell pellet was resuspended in 100 mM phosphate buffer (pH 6.4) with 20 mM NaCl and recentrifuged. EPS was precipitated from the supernatant of the first and second centrifugations with 2 volumes of cold 95% ethanol overnight at 4°C. After the precipitated material was centrifuged at $10,000 \times g$ at 4°C for 30 min, the pellet was partially resuspended in water and precipitated again with ethanol (2 volumes). This procedure was repeated twice more, after which the material was dried under a vacuum. EPS solubility was tested separately at 1 mg/ml in water, 10% NaOH (protocol for ivory nut mannan solubilization; Megazyme, Sydney, Australia), and dimethylsulfonic acid, with and without ultrasonication. Samples were hydrolyzed with 3 N HCl for 18 h before compositional analysis. Phosphorus (Sigma Chemical Co.) and sulfate (12) contents were also evaluated. Degradation by the β -mannanase from *Thermotoga neapolitana* 5068 (18) was evaluated by the Somogyi reducing-sugar assay (62) after incubation of the EPS in 900 μl of H₂O and 100 μl of enzyme at 90°C for 2 h. Samples for quantification of EPS production under different growth conditions were obtained by ethanol precipitation of the supernatant from the first cell spin and drying of the resulting material. Samples were then hydrolyzed with 3 N HCl for 18 h and neutralized with NaOH. HPLC analysis was performed on hydrolyzed EPS with a Waters high-performance carbohydrate column (4.6 by 250 mm) with a corresponding guard column at

TABLE 1. Effect of amino acid elimination on growth of *T. litoralis*

Effect of amino acid omission	Omitted amino acid	Maximum cell density (10 ⁸ cells/ml)	Doubling time (min)
None (control)	None	1.0	77
Similar to control (cell density and growth rate)	Glu	1.0	75
	Ala	1.0	80
	Asn	1.2	80
	Leu	0.8	82
	Arg	1.0	86
	Asp	1.0	92
	Gln	1.4	100
Change in growth rate	Cys	0.7	121
	His	1.0	143
	Tyr	0.8	212
	Ser	1.0	220
	Trp	1.0	220
	Pro	0.9	225
	Phe	1.0	237
	Lys	0.9	256
Change in cell density and growth rate	Gly	0.5	200
	Ile	0.5	370
	Thr	0.3	300
	Val	0.3	520

30°C, a 1.4-ml/min flow of acetonitrile-water (75:25) and a Shimadzu refractive index detector (RID-6A). Gel permeation chromatography was performed with the same instrument utilizing 7.8- by 300-mm Waters Ultrahydrogel 120, 500, and 200 columns with an Ultrahydrogel guard column and a 0.8-ml/min flow of 0.1 M sodium nitrate at 45°C. Molecular weight determinations were based on pullulan standards.

RESULTS

Defined-medium development. An objective of this study was to develop a defined sulfur-free medium for *T. litoralis* which supported growth rates and cell yields comparable to those obtained on complex media. Initially, a medium containing 2 g of maltose and 0.2 g of each of the 20 amino acids per liter was used to evaluate the effect of tryptone and yeast extract. Tryptone was eliminated first, since it did not significantly affect growth; a three-fold increase in cell density was attained when 0.05 g of yeast extract per liter was used instead of 0.05 g of tryptone per liter. Doubling the concentration of yeast extract to 0.1 g/liter in *T. litoralis* cultures did not significantly improve cell densities. Thus, a sulfur-free medium containing 0.05 g of yeast extract per liter was chosen as the basis for the amino acid elimination study.

Growth on amino acids was examined with a medium containing 0.05 g of yeast extract 2 g of maltose, and 0.1 g of each of 19 amino acids (Table 1). The effect of each amino acid was determined by comparing growth rates and maximum cell densities with the control, which contained all 20 amino acids. When Gly, Ile, Thr, or Val was eliminated, a reduction in cell density (greater than 50%) and increase in doubling time was noted relative to the control. However, *T. litoralis* was not sustainable on a medium containing only Gly, Ile, Thr, Val, or the combination of all four. Growth comparable to that of the control could be attained only with a medium containing 16 amino acids. Specific amino acids could be left out individually or in certain combinations; however, omitting any others beside Ala, Asn, Gln, or Glu resulted in decreases in cell densities and/or growth rates, sometimes to significant extents. For example, the additional omission of methionine resulted in

TABLE 2. Effect of nucleotide bases on growth of *T. litoralis*

Added nucleotide base(s) ^a	Maximum cell density (10 ⁸ cells/ml)
None	0.1
U	0.2
A, C	0.2
A, T	0.8
A, U	1.1
A, C, G, T, U	1.1

^a Base medium contains 0.2 g of 16 amino acids per liter (minus Ala, Asn, Gln, and Glu).

10-fold-lower cell densities (data not shown). The subsequent addition of 0.01 g of adenine and uracil per liter to this formulation eliminated the need for yeast extract (Table 2). The resulting defined medium, RDM, included 0.2 g of 16 amino acids and 0.01 g of adenine and uracil per liter (Table 3).

Several factors were found to significantly affect cell growth. In media containing 5 g of maltose per liter, cell densities more than doubled when amino acid levels were increased from 0.05 g of amino acids per liter to 0.1 g/liter and tripled when

TABLE 3. Composition of the defined medium for *T. litoralis*

Component	Concn (mg/liter)
Base medium	
MgCl ₂ · 6H ₂ O	1,000
Na ₂ SO ₄	1,000
NaCl	15,000
CaCl ₂ · 2H ₂ O	75
KCl	350
NaBr	50
H ₃ BO ₃	20
KI	20
SrCl ₂ · 6H ₂ O	10
Na ₂ WO ₄ · 2H ₂ O	3
K ₂ HPO ₄	140
Rezasurin	1
Na ₂ S · 9H ₂ O	100
Trace elements	
Nitritotriacetate	1.5
FeSO ₄ · 7H ₂ O	1.4
MnSO ₄ · 7H ₂ O	0.5
CoSO ₄ · 7H ₂ O	0.36
NiCl ₂ · 6H ₂ O	0.20
Na ₂ MoO ₄ · 2H ₂ O	0.001
CuSO ₄ · 5H ₂ O	0.001
Vitamins	
Pyridoxine-HCl	0.1
p-Aminobenzoic acid	0.05
Nicotinic acid	0.05
DL-Ca-pantothenate	0.05
Thiamine-HCl	0.05
DL-6,8-Thioctic acid	0.05
Riboflavin	0.04
Biotin	0.02
Folic acid	0.02
Vitamin B ₁₂	0.001
Amino acids: 16 (all except Ala, Asn, Gln, Glu)	200
Nucleotide bases	
Adenine	10
Uracil	10

FIG. 1. Effect of amino acid concentration on *T. litoralis* growth when supplemented with excess maltose (5 g/liter).

levels were further increased to 0.2 g/liter (Fig. 1). Higher levels of amino acids were not used because of solubility difficulties. When inocula from RDM cultures (0.2 g of amino acids per liter) were used, cells grew to almost a three-fold-lower density (4×10^7 cells per ml) in the presence of RDM plus NH_4Cl (1 g/liter), a trend also found for *P. furiosus* (61). Cell densities were 50% lower in cultures grown in RDM minus vitamins and trace elements (7×10^7 cells per ml), but a 15% decrease in doubling time and 40% higher cell density resulted from addition of selenium.

A variety of potential carbon and/or energy sources were added to RDM to determine their effects on *T. litoralis* growth (Table 4). Minimal levels (0.05 g/liter) of amino acids were used to simplify determination of significant improvements in cell densities by each potential substrate. Growth stimulation was found in the following order, from greatest to least: starch (11-fold), maltose (8.8-fold), sucrose (6.5-fold), pyruvate (5.9-fold), glycine (5.9-fold), mannose (4.4-fold), cellulose (2.5-fold), cellobiose (2.2-fold), galactose (1.8-fold), and glycerol (1.2-fold). An inhibitory effect was found for xylose, arabinose, and glucose. Growth was also adversely affected by supplementing the minimal medium (0.05 g of amino acids per liter) with excess (5-g/liter) isoleucine and valine. Gly, Ile, and Val were tested due to the strong negative effect of their omission in the amino acid elimination study.

Since maltose is typically used as a saccharide source for other hyperthermophiles (e.g., *P. furiosus* [58]) and *T. litoralis* grows well in its presence (11), it was selected for further studies. Slightly lower doubling times were obtained for *T. litoralis* on RDM in the presence of maltose, as shown by Fig. 2. However, addition of yeast extract did not result in improved growth.

Also evaluated were the effects of sulfur and H_2 on *T. litoralis* growth. Addition of elemental sulfur at 10 g/liter (data not shown) or colloidal sulfur, in excess or in amounts limiting for *P. furiosus* (<1.4 g/liter [10]), did not significantly affect cell growth in any medium formulations. Doubling times of approximately 50 min and maximum densities of 3×10^8 cells per ml were routinely obtained. A hydrogen atmosphere did not affect growth rates or cell densities of *T. litoralis* in the

presence or absence of maltose, yeast extract, or sulfur (data not shown).

Polysaccharide characterization. EPS was produced in batch cultures by *T. litoralis* grown on either defined or complex medium and in the presence or absence of sulfur. HPLC analysis of the EPS produced in different media reveals similar levels of mannose (no other monosaccharides were detected) under all conditions tested. Polysaccharide formation was also observed as a white filamentous material in a continuous culture grown on complex medium at a dilution rate of 0.15 h^{-1} (data not shown). The EPS produced by *T. litoralis* is mostly insoluble in water; the water-soluble fraction has an average molecular weight of approximately 41,000, as determined by gel permeation chromatography. The EPS was also insoluble in 10% NaOH and 100% dimethylsulfonic acid even with ultrasonication. Analysis of the acid-hydrolyzed polysaccharide from *T. litoralis* showed mannose as the only monosaccharide present in the polymer. The polymer was also hydrolyzed by the β -mannanase isolated from *Thermotoga neapolitana* (18), further supporting its identification as a mannan. Acid-hydrolyzed *P. furiosus* EPS also yielded a single peak when subjected to HPLC analysis. This peak, which has yet to be identified, is consistent with a mannose. Based on the initial dry weight of material, 1 to 2% sulfate and 1.5 to 4.5% phosphorus were found in three separate preparations of *T. litoralis* EPS, compared to 0.4 and 0.1% sulfate and phosphorus, respectively, in *P. furiosus* EPS.

Biofilm formation. *T. litoralis* formed a biofilm on hydrophilic surfaces under a variety of conditions. Cell adhesion on polycarbonate filters, routinely followed by epifluorescent microscopy, reached high levels when maltose and/or yeast extract was added, while only minimal attachment occurred in cultures grown on RDM alone. The highest cell levels were reached on filters incubated in a medium containing both

TABLE 4. Nutrient effects on growth of *T. litoralis*

Added compounds ^a	Cell density (10^6 cells/ml)	Fold difference ^b
None (control)	3.4	1
Complex carbohydrates		
Cellulose	8.4	2.5
Starch	38	11
Disaccharides		
Cellobiose	7.4	2.2
Sucrose	22	6.5
Maltose	30	8.8
Monosaccharides		
Glucose	1.0	0.3
Xylose	2.0	0.6
Arabinose	3.0	0.9
Mannose	15	4.4
Galactose	6.0	1.8
Amino acids		
Isoleucine	0.60	0.18
Glycine	20	5.9
Valine	3.0	0.9
Other compounds		
Pyruvate	20	5.9
Glycerol	4.0	1.2

^a Base media contain 0.05 g of 16 amino acids and 5 g of nutrient per liter except for pyruvate (1 g/liter) and glycerol (5% [vol/vol]).

^b Compared with control value, which was set at 1.

FIG. 2. Growth of *T. litoralis* in RDM (0.2 g of 16 amino acids per liter). (A) RDM; (B) RDM plus 1 g of yeast extract per liter; (C) RDM plus 2 g of maltose per liter. Results for duplicate cultures are shown. Td, doubling time; $1E+9$, 10^9 (other values are reported similarly).

yeast extract and maltose (Fig. 3A). *P. furiosus* cells also attached to polycarbonate filters when grown with or without sulfur, but in this case, more cells adhered in the presence of sulfur, possibly due to the higher cell densities reached in the aqueous suspension (data not shown). Light microscopy techniques revealed the presence of polymeric material in which the cells were bound. Biofilms formed on glass slides immersed in cultures grown in RDM with the addition of maltose, yeast extract, and sulfur in various combinations (Fig. 3B). The thin coating that formed retained Congo red stain (1). HPLC analysis of the acid-hydrolyzed biofilm indicates large amounts of mannose, as is found with the EPS isolated from this organism. Polysaccharide levels in biofilms formed under different growth conditions (medium with maltose; medium with yeast extract; medium with maltose and yeast extract; and medium

with maltose, yeast extract, and sulfur) were similar; however, significantly less material adhered to glass slides immersed in RDM alone. Scanning electron microscopy of the *T. litoralis* biofilm formed in the presence of 5 g of maltose per liter showed a high concentration of cells on the filters (Fig. 3C) but no evidence of the polymer that could be seen for *P. furiosus* (9). Samples from liquid suspensions of cells grown under the same conditions also failed to show any connective material; these results may be due to the severe dehydration steps which are used in sample preparation for electron microscopy.

FIG. 3. *T. litoralis* biofilm formation. Surfaces were immersed in cultures which were then grown 26 h in the defined medium plus 2 g of maltose and 1 g of yeast extract per liter. (A) Epifluorescent microscopy of cells attached to polycarbonate filter. Bar, 60 μ m. (B) Congo red-stained polysaccharide coating of biofilm formed on glass slide (torn with tweezers at top). Bar, 10 μ m. (C) Scanning electron microscopy of cells in biofilm on a polycarbonate filter grown as described above but in the absence of yeast extract. Bar, 5 μ m.

TABLE 5. Growth characteristics of *Thermococcales* on various media

Organism (reference)	Medium component(s) (concn) ^a	Additional component(s) ^b	Cell density (10 ⁸ cells/ml)	Doubling time (min)
<i>Pyrococcus</i> sp. strain GB-D (24, 30)	20 AA (0.1), S ⁰	TE, V	0.7	NR ^c
	YE (3), Trypticase (3), glucose (3), S ⁰	TE, V	3.0	36
<i>Desulfurococcus</i> sp. strain SY (24)	20 AA (0.1), S ⁰	TE, V	0.25	NR
	YE (2)	TE, V	1	NR
<i>P. abyssi</i> (69)	His, Thr, Arg, Tyr, Met, Val, Phe, Ile, Leu (all at 0.1)		5	40
	YE (1), PEP (4)		9	40
ES4 (52)	YE (5), PEP, S ⁰	V	3	59
	L-Glutamic acid, AA, S ⁰	V	0.6	350
	Starch, AA, S ⁰	V	0.4	300
<i>Thermococcus</i> sp. strain KS-1 (23)	YE, S ⁰		3.2	25
	20 AA, S ⁰		0.8	NR
	Glucose, 20 AA, S ⁰		2.3	NR
	Maltose, 20 AA, S ⁰		NG ^d	NR
<i>Thermococcus</i> sp. strain KS-2 (23)	YE, S ⁰		1.7	35
	AA, S ⁰		1.6	NR
	Glucose, 20 AA, S ⁰		1.3	NR
	Maltose, 20 AA, S ⁰		NG	NR
<i>Thermococcus</i> sp. strain KS-8 (23)	YE (2), S ⁰		1.4	NR
	AA		0.7	NR
	Glucose, 20 AA		2.5	NR
	Maltose, 20 AA		NG	NR
<i>T. litoralis</i> (this work)	16 AA (0.2)	A, U, TE, V	1.0	55
	16 AA (0.2), maltose (2)	A, U, TE, V	2.6	43
	16 AA (0.2), maltose (2), YE (1)	A, U, TE, V	2.6	52

^a AA, amino acids; YE, yeast extract; PEP, peptone. Concentrations are in grams per liter.

^b TE, trace elements; V, vitamins; A, adenine (0.01 g/liter); U, uracil, (0.01 g/liter).

^c NR, not reported.

^d NG, no growth.

DISCUSSION

Detailed physiological studies, which follow metabolic pathways and allow determination of bioenergetic parameters, are facilitated by the availability of defined growth media. The defined medium developed here for *T. litoralis* allows growth comparable to that obtained in complex media. In this medium, only four amino acids (Ala, Asn, Gln, and Glu) could be omitted without detrimental effects to cell densities or growth rates. This is expected, since asparagine and glutamine are rapidly deaminated at elevated temperatures (68) and since alanine and glutamate may be produced by the cells, as has been observed in *P. furiosus* (3, 15, 35, 43, 57). Development of defined media for other heterotrophic hyperthermophiles has led to varied results (Table 5). *Pyrococcus* sp. strain GB-D (24, 30), *Pyrococcus abyssi* (69), ES4 (52), and *Desulfurococcus* sp. strain SY (24) did not grow as well in defined media; however, *Thermococcus* sp. strains KS-1, KS-2, and KS-8 reached comparable maximum cell densities in both defined and complex media (23).

Sulfur reduction, required by most hyperthermophiles for growth (6, 32), has been proposed as a mechanism for the removal of inhibitory levels of H₂ and as an energetic process for the cells (32, 58). *Thermococcus celer*, *T. peptonophilus*, and *P. furiosus* are significantly stimulated by the presence of S⁰ (21, 58, 73), while *Thermococcus chitonophagus* is slightly stimulated (27). Only a few studies have reported the effects of H₂ on cell growth; *T. chitonophagus*, *Thermococcus stetteri*, and

P. furiosus are all strongly inhibited by H₂ (27, 41, 53) under certain conditions. S⁰ stimulation and H₂ inhibition in *P. furiosus* depend greatly on the presence of maltose (41, 58) for unknown reasons. *T. litoralis* differs from these organisms in that it was not affected by the presence of sulfur or H₂ under conditions tested, including the absence of maltose. The ineffectiveness of S⁰ contradicts previous reports (7, 48). This discrepancy is possibly a result of acclimation of the cells in this study by repeated transfer of *T. litoralis* into sulfur-free media prior to growth experiments. The lack of sulfur stimulation may be due to the presence of other mechanisms for processing reducing equivalents, such as production of alcohols (39) or alanine (35, 57).

The production of polysaccharides by extremophiles and archaea has been investigated only to a limited extent (22, 38, 49, 55, 63). Extracellular polysaccharides have been identified in hydrothermal vent bacteria (22, 55) and in some archaea such as halobacteria (4), methanogens (63), and sulfur-utilizing, autotrophic acidophiles (49). The EPS produced by *T. litoralis* apparently contains mannose as the only monosaccharidic constituent. Production of a mannan-like compound by a prokaryote is interesting, since mannans are typically produced by eukaryotes, such as plants or yeasts (45). The excellent growth of *T. neapolitana* (8) on galactomannans (44) and the production of both endo- and exo-acting β -mannanases (18) may indicate that certain members of hyperthermophilic communities are able to utilize *T. litoralis* EPS as a carbon and

energy source. Sulfated EPSs are common in all domains of life, including archaea (4, 49), eucarya (26), and bacteria (22, 50, 65). These EPSs, such as sulphoevernan, chondroitin sulfate, dextran sulfate, heparin, and mannan sulfate, protect eukaryotic cells from viruses, including human immunodeficiency virus type 1, by inhibiting virus particle adsorption to host cells (28, 65, 70).

The adhesion of marine bacteria to surfaces, a process in which EPSs are likely to play a role, is not unusual (14, 42). This phenomenon has not been investigated to any extent in hyperthermophilic archaea, although some organisms have been isolated from living and nonliving surfaces surrounding hydrothermal vent sites (6, 16). In the archaea, attachment is a requirement in *S. acidocaldarius* for sulfur oxidation (71) and in ES4 for transport and respiration of amino acids (6). No specific information has been provided as to the composition of the adherents in these two cases. In contrast, *P. furiosus* was found to reduce elemental sulfur when physically separated from sulfur particles by utilizing polysulfides which form from S^0 (10). EPSs are implicated in the adhesion process and the formation of biofilms in bacterial systems (2, 13, 17, 20, 54). More specifically, mannans from *Candida albicans* have been found to be directly involved in adhesion (31, 47).

The great diversity of hyperthermophilic organisms discovered to date has generated interest in a variety of potential applications involving thermostable enzymes, synthesis, and degradation reactions uniquely suited to elevated temperatures. However, difficulties in isolation and cultivation have limited physiological studies and, therefore, the implementation of new technologies utilizing hyperthermophiles. The work presented in this document addresses some of the challenges inherent in this area and provides additional insight into the specific growth physiology of *T. litoralis*. Further study into biofilm development and the role of EPSs is necessary, not only to better understand the metabolic and ecological strategies of *T. litoralis* but also to assist investigations into the interactive dynamics within consortia of hyperthermophilic microorganisms (40, 59, 66). Through exploration of hyperthermophilic mixed cultures in suspension and biofilm communities, new revelations of the natural growth of these organisms, as opposed to that obtained in pure suspensions, may result (59, 66), leading to significant improvements in cultivation techniques and establishment of important biotransformations (40).

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REFERENCES

- Allison, D. G., and I. W. Sutherland. 1984. A staining technique for attached bacteria and its correlation to extracellular carbohydrate production. *J. Microbiol. Methods* 2:93-99.
- Allison, D. G., and I. W. Sutherland. 1987. The role of exopolysaccharides in adhesion of freshwater bacteria. *J. Gen. Microbiol.* 133:1319-1327.
- Andreotti, G., M. V. Cubellis, G. Nitti, G. Sanna, X. Mai, G. Marino, and M. W. W. Adams. 1994. Characterization of aromatic aminotransferases in the hyperthermophilic archaeon *Thermococcus litoralis*. *Eur. J. Biochem.* 220:543-549.
- Antón, J., I. Meseguer, and F. Rodríguez-Valera. 1988. Production of an extracellular polysaccharide by *Haloferax mediterranei*. *Appl. Environ. Microbiol.* 54:2381-2386.
- Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc. Natl. Acad. Sci. USA* 91:1609-1613.
- Baross, J. A., and J. W. Deming. 1995. Growth at high temperatures: isolation and taxonomy, physiology, and ecology, p. 169-217. In D. M. Karl (ed.), *The microbiology of deep-sea hydrothermal vents*. CRC Press, Boca Raton, Fla.
- Belkin, S., and H. W. Jannasch. 1985. A new extremely thermophilic, sulfur-reducing heterotrophic, marine bacterium. *Arch. Microbiol.* 141:181-186.
- Belkin, S., C. O. Wirsén, and H. W. Jannasch. 1986. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. *Appl. Environ. Microbiol.* 51:1180-1185.
- Blumentals, I. I., S. H. Brown, R. N. Schicho, A. K. Skaja, H. R. Constantino, and R. M. Kelly. 1990. The hyperthermophilic archaeobacterium, *Pyrococcus furiosus*: development of culturing protocols, perspectives on scaleup, and potential applications. *Ann. N. Y. Acad. Sci.* 589:301-314.
- Blumentals, I. I., M. Itoh, G. J. Olson, and R. M. Kelly. 1990. Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Appl. Environ. Microbiol.* 56:1255-1262.
- Brown, S. H., and R. M. Kelly. 1993. Characterization of amylolytic enzymes, having both α -1,4 and α -1,6 hydrolytic activity, from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. *Appl. Environ. Microbiol.* 59:2614-2621.
- Carney, S. L. 1986. Proteoglycans, p. 136-137. In M. F. Chaplin and J. F. Kennedy (ed.), *Carbohydrate analysis: a practical approach*. IRL Press, Oxford.
- Christensen, B. E. 1989. The role of extracellular polysaccharides in biofilms. *J. Biotechnol.* 10:181-202.
- Corpe, W. A. 1970. Attachment of marine bacteria to solid surfaces, p. 73-87. In R. S. Manly (ed.), *Adhesion in biological systems*. Academic Press, New York.
- De Castele, M. V., M. Demarez, C. Legrain, N. Glansorff, and A. Piérard. 1990. Pathways of arginine biosynthesis in extreme thermophilic archaeo- and eubacteria. *J. Gen. Microbiol.* 136:1177-1183.
- Deming, J. W., and J. A. Baross. 1993. Deep-sea smokers: windows to a subsurface biosphere? *Geochim. Cosmochim. Acta* 57:3219-3230.
- Denyer, S. D., G. W. Hanlon, and M. C. Davies. 1993. Mechanisms of adherence, p. 13-27. In S. P. Denyer, S. P. Gorman, and M. Sussman (eds.), *Microbial biofilms: formation and control*. Blackwell Scientific Publications, Oxford.
- Duffaud, G. D., C. M. McCutchen, P. Leduc, K. N. Parker, and R. M. Kelly. 1990. Purification and characterization of extremely thermostable β -mannanase, β -mannosidase, and α -galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. *Appl. Environ. Microbiol.*, in press.
- Fiala, G., and K. O. Stetter. 1986. *Pyrococcus furiosus*, sp. nov., represents a novel genus of marine heterotrophic archaeobacteria growing optimally at 100°C. *Arch. Microbiol.* 145:56-61.
- Fletcher, M., and G. D. Floodgate. 1973. An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. *J. Gen. Microbiol.* 74:325-334.
- González, J. M., C. Kato, and K. Horikoshi. 1995. *Thermococcus peptonophilus* sp. nov., a fast-growing, extremely thermophilic archaeobacterium isolated from deep-sea hydrothermal vents. *Arch. Microbiol.* 164:159-164.
- Guezennec, J. G., P. Pignet, G. Raguene, E. Deslandes, Y. Lijour, and E. Gentic. 1994. Preliminary chemical characterization of unusual eubacterial exopolysaccharides of deep-sea origin. *Carbohydr. Polym.* 24:287-294.
- Hoaki, T., M. Nishijima, M. Kato, K. Adachi, S. Mizobuchi, N. Hanzawa, and T. Maruyama. 1994. Growth requirements of hyperthermophilic sulfur-dependent heterotrophic archaea isolated from a shallow submarine geothermal system with reference to their essential amino acids. *Appl. Environ. Microbiol.* 60:2898-2904.
- Hoaki, T., C. O. Wirsén, S. Hanzawa, T. Maruyama, and H. W. Jannasch. 1993. Amino acid requirements of two hyperthermophilic archaeal isolates from deep-sea vents, *Desulfurococcus* strain SY and *Pyrococcus* strain GB-D. *Appl. Environ. Microbiol.* 59:610-613.
- Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 33:1225-1228.
- Hronowski, L., and T. P. Anastassiades. 1980. The effect of cell density on net rates of glycosaminoglycan synthesis and secretion by cultured rat fibroblasts. *J. Biol. Chem.* 255:10091-10099.
- Huber, R., J. Stöhr, S. Hohenhaus, R. Rachel, S. Burggraf, H. W. Jannasch, and K. O. Stetter. 1995. *Thermococcus chitonophagus* sp. nov., a novel, chitin-degrading, hyperthermophilic archaeum from a deep-sea hydrothermal vent environment. *Arch. Microbiol.* 164:255-264.
- Ito, M., M. Baba, K. Hirabayashi, T. Matsumoto, M. Suzuki, S. Shigeta, and E. De Clercq. 1989. In vitro activity of mannan sulfate, a novel sulfated polysaccharide, against human immunodeficiency virus type I and other enveloped viruses. *Eur. J. Clin. Microbiol. Infect. Dis.* 8:171-173.
- Jannasch, H. W., C. O. Wirsén, S. J. Molyneux, and T. A. Langworthy. 1988. Extremely thermophilic fermentative archaeobacteria of the genus *Desulfu-*

- rococcus* from deep-sea hydrothermal vents. Appl. Environ. Microbiol. **54**: 1203–1209.
30. Jannasch, H. W., C. O. Wirsén, S. J. Molyneux, and T. A. Langworthy. 1992. Comparative physiological studies on hyperthermophilic archaea isolated from deep-sea hot vents with emphasis on *Pyrococcus* strain GB-D. Appl. Environ. Microbiol. **58**:3472–3481.
 31. Kanbe, T., Y. Han, B. Redgrave, M. H. Riesselman, and J. E. Cutler. 1993. Evidence that mannans of *Candida albicans* are responsible for adherence of yeast forms to spleen and lymph node tissue. Infect. Immun. **61**:2578–2584.
 32. Kelly, R. M., and M. W. W. Adams. 1994. Metabolism in hyperthermophilic microorganisms. Antonie van Leeuwenhoek. **66**:247–270.
 33. Kelly, R. M., I. I. Blumentals, L. J. Snowden, and M. W. W. Adams. 1992. Physiological and biochemical characteristics of *Pyrococcus furiosus*, a hyperthermophilic archaeobacterium. Ann. N. Y. Acad. Sci. **665**:309–320.
 34. Kengen, S. W. M., F. A. M. De Bok, N. D. Van Loo, C. Dijkema, A. J. M. Stams, and W. M. De Vos. 1994. Evidence for the operation of a novel Embden-Meyerhof pathway that involves ADP-dependent kinases during sugar fermentation by *Pyrococcus furiosus*. J. Biol. Chem. **269**:17537–17541.
 35. Kengen, S. W. M., and A. J. M. Stams. 1994. Formation of L-alanine as a reduced end product in carbohydrate fermentation by the hyperthermophilic archaeon *Pyrococcus furiosus*. Arch. Microbiol. **161**:168–175.
 36. Klages, K. U., and H. W. Morgan. 1994. Characterization of an extremely thermophilic sulphur-metabolizing archaeobacterium belonging to the Thermococcales. Arch. Microbiol. **162**:261–266.
 37. Kobayashi, T., Y. S. Kwak, T. Akiba, T. Kudo, and K. Horikoshi. 1994. *Thermococcus profundus* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Syst. Appl. Microbiol. **17**:232–236.
 38. König, H., R. Skorko, W. Zillig, and W.-D. Reiter. 1982. Glycogen in thermocacidophilic archaeobacteria of the genera *Sulfolobus*, *Thermoproteus*, *Desulfurococcus* and *Thermococcus*. Arch. Microbiol. **132**:297–303.
 39. Ma, K., F. T. Robb, and M. W. W. Adams. 1994. Purification and characterization of NADP-specific alcohol dehydrogenase and glutamate dehydrogenase from the hyperthermophilic archaeon *Thermococcus litoralis*. Appl. Environ. Microbiol. **60**:562–568.
 40. Mack, W. N., J. P. Mack, and A. O. Ackerson. 1975. Microbial film development in a trickling filter. Microb. Ecol. **2**:215–226.
 41. Malik, B., W.-W. Su, H. L. Wald, I. I. Blumentals, and R. M. Kelly. 1989. Growth and gas production for hyperthermophilic archaeobacterium, *Pyrococcus furiosus*. Biotechnol. Bioeng. **34**:1050–1057.
 42. Marshall, K. C. 1992. Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. ASM News **58**:202–208.
 43. Martins, L. O., and H. Santos. 1995. Accumulation of mannosylglycerate and di-myo-inositol-phosphate by *Pyrococcus furiosus* in response to salinity and temperature. Appl. Environ. Microbiol. **61**:3299–3303.
 44. McCutchen, C. M., G. D. Duffaud, P. Leduc, A. R. H. Peterson, A. Tayal, S. A. Khan, and R. M. Kelly. 1996. Characterization of extremely thermostable enzymatic breakers (α -1,6-galactosidase and β -1,4-mannanase) from the hyperthermophilic bacterium *Thermotoga neopolitana* 5068 for hydrolysis of guar gum. Biotechnol. Bioeng. **52**:332–339.
 - 44a. McDowell, E. M., and B. F. Trump. 1976. Histologic fixative suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. **100**:405–414.
 45. Millane, R. P., and T. L. Hendrixson. 1994. Crystal structures of mannan and glucomannans. Carbohydr. Polym. **25**:245–251.
 46. Miroshnichenko, M. L., E. A. Bonch-Osmolovskaya, A. Neuner, N. A. Kostrikina, N. A. Chernych, and V. A. Alekseev. 1989. *Thermococcus stetteri* sp. nov., a new extremely thermophilic marine sulfur-metabolizing archaeobacterium. Syst. Appl. Microbiol. **12**:257–262.
 47. Miyakawa, Y., T. Kuribayashi, K. Kagaya, M. Suzuki, T. Nakase, and Y. Fukazawa. 1992. Role of specific determinants in mannan of *Candida albicans* serotype A in adherence to human buccal epithelial cells. Infect. Immun. **60**:2493–2499.
 48. Neuner, A., H. W. Jannasch, S. Belkin, and K. O. Stetter. 1990. *Thermococcus litoralis* sp. nov.: a new species of extremely thermophilic marine archaeobacteria. Arch. Microbiol. **153**:205–207.
 49. Nicolaus, B., M. C. Manca, I. Romano, and L. Lama. 1993. Production of an exopolysaccharide from two thermophilic archaea belonging to the genus *Sulfolobus*. FEMS Microbiol. Lett. **109**:203–206.
 50. Panoff, J.-M., B. Priem, H. Morvan, and F. Joset. 1988. Sulphated exopolysaccharides produced by two unicellular strains of cyanobacteria, *Synechocystis* PCC 6803 and 6714. Arch. Microbiol. **150**:558–563.
 51. Payne, J. W. 1980. Transport and utilization of peptides by bacteria, p. 211–256. In J. W. Payne (ed.), Microorganisms and nitrogen sources. John Wiley & Sons, Chichester, Great Britain.
 52. Pledger, R. J., and J. A. Baross. 1991. Preliminary description and nutritional characterization of a chemorganotrophic archaeobacterium growing at temperatures of up to 110°C isolated from a submarine hydrothermal vent environment. J. Gen. Microbiol. **137**:203–211.
 53. Pusheva, M. A., A. I. Slobodkin, and E. A. Bonch-Osmolovskaya. 1992. Investigation of hydrogenase activity of the extremely thermophilic archaeobacterium *Thermococcus stetteri*. Mikrobiologiya **60**:5–11.
 54. Quintero, E. J., and R. M. Weiner. 1995. Evidence for the adhesive function of the exopolysaccharide of *Hyphomonas* strain MHS-3 in its attachment to surfaces. Appl. Environ. Microbiol. **61**:1897–1903.
 55. Raguene, G., P. Pignet, G. Gauthier, A. Peres, R. Christen, H. Gougeaux, G. Barbier, and J. Guezennec. 1996. Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, *Alteromonas macleodii* subsp. *fijiensis*, and preliminary characterization of the polymer. Appl. Environ. Microbiol. **62**:67–73.
 56. Robinson, R. W., H. C. Aldrich, S. F. Hurst, and A. S. Bleiweis. 1985. Role of the cell surface of *Methanosarcina mazei* in cell aggregation. Appl. Environ. Microbiol. **49**:321–327.
 57. Schäfer, T., K. B. Xavier, H. Santos, and P. Shönheit. 1994. Glucose fermentation to acetate and alanine in resting cell suspensions of *Pyrococcus furiosus*: proposal of a novel glycolytic pathway based on ¹³C labeling data and enzyme activities. FEMS Microbiol. Lett. **121**:107–114.
 58. Schicho, R. N., K. Ma, M. W. W. Adams, and R. M. Kelly. 1993. Bioenergetics of sulfur reduction in the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **175**:1823–1830.
 59. Siebel, M. A., and W. G. Characklis. 1991. Observations of binary population biofilms. Biotechnol. Bioeng. **37**:778–789.
 60. Smith, P. F., T. A. Langworthy, and M. R. Smith. 1975. Polypeptide nature of growth requirement in yeast extract for *Thermoplasma acidophilum*. J. Bacteriol. **124**:884–892.
 61. Snowden, L. J., I. I. Blumentals, and R. M. Kelly. 1992. Regulation of proteolytic activity in the hyperthermophile *Pyrococcus furiosus*. Appl. Environ. Microbiol. **58**:1134–1141.
 62. Somogyi, M. 1952. Notes on sugar determination. J. Biol. Chem. **195**:19–23.
 63. Sowers, K. R., and R. P. Gunsalus. 1988. Adaptation for growth at various saline concentrations by the archaeobacterium *Methanosarcina thermophila*. J. Bacteriol. **170**:998–1002.
 64. Stetter, K. O. 1995. Microbial life in hyperthermal environments. ASM News **61**:285–290.
 65. Sudo, H., J. G. Burgess, H. Takemasa, N. Nakamura, and T. Matsunaga. 1995. Sulfated exopolysaccharide production by the halophilic cyanobacterium *Aphanocapsa halophytia*. Curr. Microbiol. **30**:219–222.
 66. Thiele, J. H., and J. G. Zeikus. 1988. Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. Appl. Environ. Microbiol. **54**:20–29.
 67. Usenko, I. A., L. O. Severina, and V. K. Plakunov. 1993. Uptake of sugars and amino acids by extremely thermophilic archae- and eubacteria. Microbiology **62**:272–277.
 68. Völkin, D. B., and A. M. Klivanov. 1990. Minimizing protein inactivation, p. 1–24. In T. E. Creighton (ed.), Protein function: a practical approach. IRL Press, Oxford.
 69. Watrin, L., V. Martin-Jezequel, and D. Prieur. 1995. Minimal amino acid requirements of the hyperthermophilic archaeon *Pyrococcus abyssi*, isolated from deep-sea hydrothermal vents. Appl. Environ. Microbiol. **61**:1138–1140.
 70. Weiler, B. E., H. C. Schröder, V. Stefanovich, D. Stewart, J. M. S. Forrest, L. B. Allen, B. J. Bowden, M. H. Kreuter, R. Voth, and W. E. G. Müller. 1990. Sulphoevernan, a polyanionic polysaccharide, and the narcissus lectin potentially inhibit human immunodeficiency virus infection by binding to viral envelope protein. J. Gen. Virol. **71**:1957–1963.
 71. Weiss, B. L. 1973. Attachment of bacteria to sulphur in extreme environments. J. Gen. Microbiol. **77**:501–507.
 - 71a. White, C. A., and J. F. Kennedy. 1986. Oligosaccharides, p. 38. In M. F. Chaplin and J. F. Kennedy (ed.), Carbohydrate analysis: a practical approach. IRL Press, Oxford, United Kingdom.
 72. Zillig, W., I. Holz, D. Janekovic, H.-P. Klenk, E. Imself, J. Trent, S. Wunderl, V. H. Forjaz, R. Coutinho, and T. Ferreira. 1990. *Hyperthermus butylicus*, a hyperthermophilic sulfur-reducing archaeobacterium that ferments peptides. J. Bacteriol. **172**:3959–3965.
 73. Zillig, W., I. Holz, D. Janekovic, W. Schäfer, and W. D. Reiter. 1983. The archaeobacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaeobacteria. Syst. Appl. Microbiol. **4**:88–94.