

Effects of trace element concentrations on culturing thermophiles

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Abstract The majority of microorganisms in natural environments resist laboratory cultivation. Sometimes referred to as ‘unculturable’, many phylogenetic groups are known only by fragments of recovered DNA. As a result, the ecological significance of whole branches of the ‘tree of life’ remains a mystery; this is particularly true when regarding genetic material retrieved from extreme environments. Geochemically relevant media have been used to improve the success of culturing Archaea and Bacteria, but these efforts have focused primarily on optimizing pH, alkalinity, major ions, carbon sources, and electron acceptor–donor pairs. Here, we cultured thermophilic microorganisms from ‘Sylvan Spring’ (Yellowstone National Park, USA) on media employing different trace element solutions, including one that mimicked the source fluid of the inocula. The growth medium that best simulated trace elements found in ‘Sylvan Spring’ produced a more diverse and faster growing mixed culture than media containing highly elevated trace element concentrations. The elevated trace element medium produced fewer phylotypes and inhibited growth. Trace

element concentrations appear to influence growth conditions in extreme environments. Incorporating geochemical data into cultivation attempts may improve culturing success.

Keywords Culturing · Thermophiles · Trace element composition · Hydrothermal systems · Microbial ecology

Introduction

Only a small percentage of known Archaea and Bacteria have been cultured, isolated, and at least partially characterized. Surveys of environmental 16S rRNA gene sequences from waters, soils, sediments, biofilms, and other biotopes indicate a far greater diversity than what exists in the culture collections (Rappé and Giovannoni 2003; Schleper et al. 2005; Handelsman and Tiedje 2007); recent estimates indicate that uncultured phyla still outnumber phyla with cultured representatives (M. Rappé, pers. comm.). Efforts that examine the in situ ecological roles of uncultured organisms in natural environments yield helpful, but limited results (Fishbain et al. 2003; Boyd et al. 2009). For example, environmental conditions may be isolated, controlled for, and manipulated in culturing approaches, whereas this is difficult or impossible in natural systems (particularly those that are protected, such as Yellowstone National Park). There are phylogenetically significant groups of Archaea and Bacteria from which no cultured representatives have been retrieved (Giovannoni et al. 1995; Takai and Horikoshi 1999; Takai and Sako 1999; Ochsenreiter et al. 2003; Rappé and Giovannoni 2003). Owing to difficulties in obtaining pure cultures (Adams 1995; Huber et al. 1995; Burggraf et al. 2001), many organisms defined exclusively by 16S rRNA sequences

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have been labeled as “unculturable” (Barns et al. 1994; Hugenholtz et al. 1998). These uncultured organisms may represent minor constituents of complex microbial communities or organisms that grow as obligatory symbionts in a mixed culture. However, they may also represent major or ecologically important organisms that have not been provided with suitable growth conditions in the laboratory (Huber et al. 1995, 2002).

Traditional enrichment techniques can be quite effective, particularly when used at ambient or slightly elevated temperatures (Guirard and Snell 1981; Kreig 1981; Robb et al. 1995; Burlage et al. 1998) and are often successful in producing pure strains that represent the dominant species in the inoculum, or the most capable of surviving the growth conditions provided (Burnett et al. 1957; Kreig 1981; Ward et al. 2006; Caldwell et al. 2002). The challenges of culturing from thermal habitats have long been recognized (Castenholtz 1988a, b; Robb et al. 1995). Some enlightened efforts to culture thermophiles sometimes used filtered hot spring fluid (Brock and Freeze 1969; D’Imperio et al. 2008) or used analysis of in situ chemistry to develop a basal salts medium for culturing thermophiles (Castenholtz 1969; Brierley and Brierley 1973; Huber et al. 1998a, b). However, the majority of early attempts to cultivate thermophiles made use of traditional growth media or trace element solutions, such as “Medium D”, “Allen’s Medium”, or “SME Medium” (Allen 1959; Castenholtz 1969; Stetter et al. 1983; Seeger et al. 1986; Castenholtz 1988a, b; Boone et al. 1989; Nakagawa et al. 2005; Boyd et al. 2007) that may have had limited similarity to the host environments of the target organisms (Guirard and Snell 1981; Kreig 1981). In particular, few records can be found of trace element (or trace mineral) solutions based on thermal fluid chemistry (though see Amend et al. 2003a, b; Osburn and Amend 2010), and typically a traditional recipe such as “Wolfe’s Trace Element Solution” or “Trace Element Solution SL-7” is used (Wolin et al. 1963; Atlas 2004). This practice is continued in modern attempts to culture thermophiles (Eder and Huber 2002; Gotz et al. 2002; Kashefi et al. 2002; Haouari et al. 2008). New culturing techniques specifically targeting the ‘unculturables’ have been developed that vary nutritional and environmental parameters (Johnson 1995; Kashefi et al. 2002; Rappé et al. 2002; Amend et al. 2003a; Edwards et al. 2003; Cho and Giovannoni 2004; Davis et al. 2005; Köpke et al. 2005; Lloyd et al. 2005; Osburn and Amend 2010). In addition, sophisticated methods may be used to physically separate individual cells or colonies from the mixed community (Huber et al. 1995; Huber et al. 1998a, b; Kaeberlein et al. 2002; Zengler et al. 2002).

The chemistry of a growth medium is vital to the success of culturing attempts. There is plentiful evidence that terrestrial hydrothermal ecosystems boast a wide range of chemical compositions, even within the same geothermal

system (Allen and Day 1935; Brock 1978; White et al. 1988; Ball et al. 1998, 2001; Amend et al. 2003a, b; Meyer-Dombard et al. 2005; Shock et al. 2005, 2010; Nordstrom et al. 2009). In the present study, we had two goals. First, we wanted to determine if trace element chemistry had an affect on the growth of thermophilic consortia from ‘Sylvan Spring’ geothermal fluid. To meet this goal, we investigated the effects of elevated trace element concentrations on the growth of ‘Sylvan Spring’ consortia (stage one of the study), and investigated the acclimatization and response of these consortia to changing trace element conditions (stage two of the study). The aqueous growth media tested differed only in their trace element chemistry and were based strictly on the in situ geochemistry of ‘Sylvan Spring’ (prior to the addition of complex carbon substrate), or were entirely independent of the trace element geochemistry of ‘Sylvan Spring’. Our second goal was to incorporate our findings in the design of growth media for various other Yellowstone environments. Geochemical data (major ions and trace elements) collected over 3 years from Yellowstone National Park (YNP) were used as a template to design aqueous growth media, including trace elements, explicitly for culturing thermophiles.

Methods

Sample collection

Thermal fluid was collected from ‘Sylvan Spring’,¹ YNP (0518359 Easting; 4949525 Northing, Universal Transverse Mercator zone 12). Fluid was collected ~3 m from the edge of the pool and ~0.5 m below the surface. Sample was placed in a sterile 175-mL glass serum bottle, sealed with a butyl stopper, and stored at 4°C. Samples of other thermal features listed in Table 1 were collected similarly.

Geochemistry

Analyses of redox-sensitive and major ions in fluid samples were conducted as reported in Shock et al. (2010). Minor and trace elements in these fluids were determined using a Finnigan MAT (Thermo Electron) Element 1 single-collector double-focusing magnetic sector inductively coupled plasma mass spectrometer (ICP-MS). Uncertainties are one standard deviation for minor (3%) and trace (5%) elements. Accuracy and precision were determined by analyses of

¹ For information regarding the naming of this hydrothermal feature, see Meyer-Dombard, et al., 2005.

Table 1 Trace element concentrations (mM) in selected YNP locations

Element (mM)	'Sylvan Spring' 000625G ^a	Sylvan area 000625F ^a	Sylvan area 000626A ^a	Sylvan area 000626C ^a	Sylvan area 000625A	Mound Spring 990724A	Obs. pool area 990720D
Easting	0518347	0518446	0518384	0518575	0518577	0511112	0544528
Northing	4949528	4949592	4949579	4949713	4949675	4934624	4939786
T (°C)	81	80.9	77.5	74.3	87.0	93.9	81
pH	5.7	4.1	4.9	6.6	2.4	8.2	5.7
Ca	0.10	0.10	0.07	0.11	5.3×10^{-2}	4.0×10^{-2}	0.58
Fe	1.1×10^{-3}	0.01	9.3×10^{-3}	1.8×10^{-4}	0.11	8.2×10^{-5}	6.2×10^{-4}
Mg	ND	2.4×10^{-4}	ND	ND	9.6×10^{-3}	3.3×10^{-4}	0.42
Co	1.7×10^{-6}	ND	ND	ND	5.1×10^{-6}	4.2×10^{-7}	bdl
Cu	7.9×10^{-6}	ND	ND	ND	1.9×10^{-5}	bdl	bdl
Al	0.013	0.08	0.28	3.8×10^{-3}	0.72	1.4×10^{-2}	8.5×10^{-3}
Zn	6.1×10^{-4}	ND	1.0×10^{-3}	ND	1.3×10^{-3}	1.2×10^{-4}	2.8×10^{-4}
Mn	4.4×10^{-4}	6.4×10^{-4}	8.7×10^{-4}	2.4×10^{-4}	1.6×10^{-3}	5.5×10^{-6}	1.2×10^{-2}
Mo	1.7×10^{-3}	1.3×10^{-3}	5.6×10^{-4}	2.0×10^{-4}	3.6×10^{-5}	2.9×10^{-4}	9.1×10^{-6}
Rb	5.6×10^{-3}	2.7×10^{-3}	1.2×10^{-3}	1.8×10^{-3}	8.5×10^{-4}	1.3×10^{-3}	1.2×10^{-3}
Sr	2.1×10^{-4}	3.2×10^{-4}	1.4×10^{-4}	2.1×10^{-4}	2.9×10^{-4}	9.8×10^{-6}	1.8×10^{-3}
V	1.8×10^{-5}	1.3×10^{-5}	2.4×10^{-5}	1.6×10^{-4}	5.2×10^{-5}	1.6×10^{-5}	9.4×10^{-6}
Pb	4.5×10^{-6}	3.4×10^{-7}	1.0×10^{-5}	bdl	1×10^{-5}	2.7×10^{-6}	5.6×10^{-7}
Ba	9.1×10^{-5}	3.1×10^{-4}	3.4×10^{-4}	1.3×10^{-4}	5×10^{-4}	9.4×10^{-6}	8.2×10^{-4}
Cd	1.7×10^{-6}	1.2×10^{-6}	1.7×10^{-6}	2.7×10^{-7}	1.1×10^{-6}	8.9×10^{-7}	1.8×10^{-7}
W	1.1×10^{-3}	8.9×10^{-4}	2.0×10^{-4}	6.3×10^{-4}	6.5×10^{-7}	1.8×10^{-3}	8.6×10^{-6}

The naming convention for sample locations is given as “yymddx”, where “yymdd” represents the date of collection, and “x” is a simple sample identifier. GPS coordinates given as Universal Transverse Mercator, zone 12

ND not determined, bdl below detection limit

^a Used in formulation of media SS1

river water standard reference materials NIST 1640 and NRC SLRS4; measured and certified values for standards were within quoted uncertainties. Sixty milliliter Nalgene sample bottles were soaked in 10% HNO₃ (trace metal grade) for 1–3 days, rinsed with deionized water, and sealed in the lab before traveling to the field. Samples were filtered through a series of 1.0, 0.8, and 0.25 μm filters and acidified to pH <2 with HNO₃. Results are reported in Table 1.

Media design

Medium SS1 was based on the in situ geochemistry of ‘Sylvan Springs’ area hydrothermal features (Meyer-Dombard 2004; Shock et al. 2010), and was used in both experiments. The remaining media incorporate “Wolfe’s trace element solution” (Wolin et al. 1963), which is independent of the trace element geochemistry of ‘Sylvan Spring’. One medium containing Wolfe’s trace element solution, hereafter referred to as medium WTE, includes the original Wolfe’s trace element solution recipe and was only used in experimental stage two. In addition, a 200× concentrated version of the WTE medium, simply known

here as medium WTE200, was used in both experimental stages. Trace element concentrations in these three media are given in Table 2.

Experimental stage one was designed to determine if elevated trace elements affected cell growth. As can be seen in Tables 1 and 2, the variability in trace elements between media SS1 and WTE often falls within the natural variation of fluids in the Sylvan Springs area. For example, concentrations of Fe in SS1 and WTE differ by one order of magnitude, but Fe varies by three orders of magnitude in the Sylvan Spring area. Similar comparisons can be made for Mg, Ca, Al, Zn, and Mo. Trace element solution WTE200 was arrived at specifically to reach concentrations that were equal to or exceeding trace element concentrations in the SS1 medium to allow us to evaluate effects of elevated trace element concentrations. In essence, the elevated trace element concentrations in medium WTE200 were chosen to eliminate doubts that may have arisen from a medium with lower trace element concentrations.

Experimental stage two further queried the acclimation and response of the stage one enrichments to changing environmental conditions. As geothermal systems in Yellowstone National Park have been shown to undergo

Table 2 Trace element concentrations (mM) in experimental and reference trace element solutions

Element (mM)	SS1	WTE	WTE200	medium D	OBP ^a	SS2 ^a	LGB ^a
Ca	0.11	0.003	0.68	0.034	0.56	0.025	0.025
Fe	3.6×10^{-4}	2.9×10^{-3}	0.59	1.7×10^{-3}	1.3×10^{-3}	0.07	5.4×10^{-5}
Mg	0.008	0.074	14.73	0.04	0.41	8.2×10^{-3}	0.25
Co	3.4×10^{-7}	2.1×10^{-3}	0.42	1.9×10^{-4}	8.5×10^{-7}	1.7×10^{-5}	1.7×10^{-6}
Cu	3.2×10^{-6}	2.0×10^{-4}	0.04	1.0×10^{-4}	2.4×10^{-6}	7.1×10^{-6}	4.7×10^{-6}
Al	0.015	1.1×10^{-4}	0.02	0.0	8.2×10^{-3}	0.86	0.012
Zn	3.5×10^{-4}	1.7×10^{-3}	0.35	1.7×10^{-3}	3.8×10^{-4}	9.6×10^{-4}	1.7×10^{-4}
Mn	4.6×10^{-4}	0.02	3.31	1.3×10^{-2}	8.7×10^{-3}	6.2×10^{-4}	1.8×10^{-5}
Mo	1.1×10^{-3}	2.1×10^{-4}	0.04	1.0×10^{-4}	2.1×10^{-5}	1.6×10^{-5}	3.2×10^{-4}
Rb	2.9×10^{-3}	0.0	0.0	0.0	1.2×10^{-3}	5.9×10^{-4}	1.3×10^{-3}
Sr	2.1×10^{-4}	0.0	0.0	0.0	1.7×10^{-3}	1.9×10^{-4}	2.3×10^{-5}
V	2.0×10^{-5}	0.0	0.0	0.0	1.2×10^{-5}	3.9×10^{-5}	1.8×10^{-5}
Pb	4.8×10^{-6}	0.0	0.0	0.0	7.2×10^{-7}	3.4×10^{-5}	4.8×10^{-6}
Ba	2.7×10^{-4}	0.0	0.0	0.0	7.7×10^{-4}	5.8×10^{-4}	2.2×10^{-5}
Cd	1.8×10^{-6}	0.0	0.0	0.0	8.9×10^{-7}	8.9×10^{-7}	8.9×10^{-7}
W	6.4×10^{-4}	0.0	0.0	0.0	1.1×10^{-5}	5.4×10^{-7}	1.7×10^{-3}

^a Trace element solutions OBP, SS2, and LGB should be used with an appropriate basal salts medium, for example as reported in Meyer-Dombard 2004. Alternatively, measured values of major ions can be found in various sources (Allen and Day 1935; Brock 1978; Ball et al. 1998, 2001; Meyer-Dombard 2004; Nordstrom et al. 2005; Shock et al. 2005, 2010). A basal salt medium for SS1 can be found in the methods of this work. Medium D given as reported in Castenholtz, 1969

drastic changes in chemistry (associated, for example, with changes in pH or dilution by increased meteoric water input) over short time periods (Nordstrom et al. 2009; Shock et al. 2010), the ability of microorganisms to cope with environmental stresses such as increasing or decreasing trace element concentrations is of interest. Here, we tested the ability of the enrichment in SS1 to acclimate to media WTE and WTE200, and the ability of the enrichment in WTE200 to acclimate to medium SS1.

Basal salt media recipes

The basal salt solution for all experimental media, including WTE and WTE200, is a pH buffered artificial ‘Sylvan Springs’ area thermal fluid; representative values of ‘Sylvan Spring’ major ion concentrations were chosen and the basal salts medium was formulated adjusting for the molecular weight of the salts added and charge balance. The ‘Sylvan Spring’ basal salts medium contains (per liter of water): 1.67 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.04 g KCl, 0.36 g NaHCO_3 , 0.27 g Na_2SO_4 , 0.5 g NaCl. All media were a yeast-sulfur (YS) formulation, and received (per L) 3 g yeast extract (Difco), which contributed additional trace elements to the media (Grant and Pramer 1962). Also added to all media were 10 mL N/P solution (containing per liter of water: 444.8 mg NH_4Cl , 34.2 mg NaNO_3 , and 83.0 mg K_2HPO_4), 0.5 mL of a 2% resazurin solution (redox indicator), and 3 g of MES (2-[N-morpholino] ethanesulfonic acid) as a pH buffer.

Trace element solution recipes

The SS1 trace element solution (Table 2) was formulated by selecting representative values (Table 1) then adjusted for the molecular weight of the salts added and charge balance. The following were added to 1 L of ‘Sylvan Spring’ basal salt solution to obtain medium SS1: 15.77 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.06 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 6.89 mg $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.09 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.07 mg $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 μg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.8 μg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.12 mg H_2WO_4 , 0.16 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 4.0 μg $\text{VOSO}_4 \cdot 3.5\text{H}_2\text{O}$, 0.1 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 μg $\text{CdSO}_4 \cdot 8/3\text{H}_2\text{O}$, 2.0 μg PbCrO_4 , 0.36 mg RbCl, and 0.06 mg $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$.

Medium WTE was obtained by adding the following to 1 L of the ‘Sylvan Spring’ basal salt solution: 0.5 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 mg $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 15 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g MnSO_4 , 0.5 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 mg $\text{CuSO}_4 \cdot 12\text{H}_2\text{O}$, 0.05 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.5 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 mg H_3BO_3 , 7.5 mg nitriooacetic acid and 5 mg NaCl (Wolin et al. 1963). Medium WTE200 was arrived at by concentrating the WTE recipe 200-fold.

Media preparation

The pH of all media was adjusted to 5.5, and the medium was autoclaved at 121°C for 40 min. Trace element solutions were then added. Anaerobic media were prepared by heating and degassing under N_2 . Acid-washed glass serum bottles containing ~ 2 g S^0 (sterilized for >3 days in an

80°C oven) were filled with 60 mL of anoxic media while under N₂. Bottles were capped with butyl-rubber stoppers, pressurized with N₂ to 3 bar, and media were reduced with 1.8 mL of a 2.5% Na₂S solution prior to inoculation. Although precipitation reactions of sulfide or sulfur within the growth media may have occurred, mineralization was not observed microscopically, and all experimental media received the same Na₂S treatment with equal potential effect on trace element concentrations. Non-experimental media were formulated in a similar fashion. Selected trace element solutions for other Yellowstone hydrothermal features are given in Table 1; full recipes for media OBP, SS2, and LGB are given in Meyer-Dombard 2004.

Inoculation/incubation

To test the response of cultures to elevated trace element concentrations, medium SS1 was used as a control against medium WTE200 in experimental stage one. Both SS1 and WTE200 media were inoculated with 1 mL of ‘Sylvan Spring’ fluid and incubated at 80°C, resulting in cultures 1A (in SS1) and 1B (in WTE200). Incubations continued for the same length of time in apparent exponential growth, as indicated by direct cell counts verifying total cell numbers doubling. The response of consortia 1A and 1B to different growth media formulations was investigated in stage two. Here, 0.5 mL of culture 1A (grown in medium SS1 in experimental stage one) was used to inoculate 60 mL of fresh WTE and WTE200 media, yielding cultures 2A and 2B, respectively. A 0.5 mL aliquot of culture 1B (grown in medium WTE200, in stage one) was introduced into 60 mL of fresh SS1 medium (culture 2C). Incubation conditions were identical to stage one.

Microscopy

Cultures were regularly observed by phase contrast and epi-fluorescence microscopy to verify cell growth, were subsampled as appropriate, and were preserved and DAPI stained for whole cell counts as described (Amend et al. 2003a). Doubling times were determined by linear regression of cell counts. Transmission electron microscopy (TEM) was done by Washington University’s Molecular Microbiology Imaging Facility. Subsamples were removed after 756 h in experimental stage one, and 449 h in experimental stage two. Samples were prepared for ultrastructural analysis as previously described (Amend et al. 2003a). Sections of 70–80 nm were cut, stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA, Inc., Peabody, MA).

DNA extraction and amplification

When total cell growth could be verified to be doubling exponentially, cultures were allowed to grow for another 800 h, at which point aliquots of both cultures from experimental stage one were removed for DNA extraction. Ten milliliters of each culture was pelleted (per extraction), and DNA was extracted by chemical extraction using the QIAamp DNA Mini Kit (QIAGEN cat# 51306), and mechanical extraction by bead-beating using the FastDNA Spin soil extraction kit (Bio101) as described (Amend et al. 2003a; Meyer-Dombard et al. 2005). The 16S ribosomal RNA gene was amplified with the polymerase chain reaction (PCR) from all 4 DNA extractions, using primers 21F and 1391R targeting Archaea, and 27F and 1492R targeting Bacteria (Lane 1991), as described (Amend et al. 2003a). A negative control sample (no added DNA) ensured that products were not contaminated by the lab procedure.

Sequencing/phylogenetic inference

Amplified 16S rRNA genes (PCR products) from extractions of cultures 1A and 1B, and the negative control were cloned as previously described (Meyer-Dombard et al. 2005). Initially, ~650 bp of each of 48 clones from each library were sequenced using primers 21F or 27F, and unique operational taxonomic units (OTUs), were identified for each library using a 95% similarity cut off. For unique OTUs, 1300–1400 bp of the 16S rRNA gene were sequenced and analyzed by phylogenetic comparison as previously reported (Meyer-Dombard et al. 2005). Sequences representing organisms in both cultures were deposited in GenBank (accession numbers EF088324–EF088326).

Results

Trace element geochemistry

The ‘Sylvan Spring’ area in YNP features a large thermal pool (‘Sylvan Spring’, see Meyer-Dombard et al. 2005) and a number of smaller thermal features of varying fluid chemistry. ‘Sylvan Spring’ is 13 × 20 m in diameter, is slightly acidic (pH ~5.5) and near boiling ($T = 85^{\circ}\text{C}$), and has a high gas flux (Meyer-Dombard 2004; Meyer-Dombard et al. 2005). Results of trace element analyses for ‘Sylvan Spring’ and selected other YNP hot springs are presented in Table 1. These data include several thermal features in the ‘Sylvan Springs’ area; four were used in the formulation of medium SS1 (000625F, 000625G, 000626A, 000626C). Also shown for comparison in Table 1 are data from an acidic feature in the ‘Sylvan

Springs' area, an alkaline chloride hot spring in the Lower Geyser Basin, and a neutral pH, gas-dominated feature in the Mud Volcano area. Among these locations, the concentrations of Co, Cu, Zn, Rb, and Cd were the least variable, while variation of three or more orders of magnitude was seen in Fe, Mg, Mn, Mo, Sr, and W.

Experimental stage one

In stage one, medium SS1 (the control), and medium WTE200 were used to investigate the effects of elevated trace element concentrations on consortia from 'Sylvan Spring'. Growth curves, diversity, and morphology are presented in the following sections.

Cell growth

Direct cell counts in mixed cultures 1A (medium SS1) and 1B (medium WTE200) as functions of time are depicted in Fig. 1. Cell densities at t_0 of cultures 1A and 1B were 4.9×10^5 and 4.4×10^5 cells/mL, respectively. It can be seen that cell densities in culture 1A increased in three distinct phases from 4.9×10^5 cells/mL at t_0 to 1.5×10^8 cells/mL after 1056 h, at which point tubes were visually turbid. Calculated generation times were ~ 42 h in the first phase (0–90 h), ~ 233 h in the second phase (90–888 h), and ~ 67 h during the final phase (>888 h). In culture 1B, cell densities increased from 4.4×10^5 cells/mL at t_0 to 1.1×10^7 cells/mL after 1256 h, and growth trends are not as well resolved. For the first 477 h (phase 0), cell densities fluctuated, showing relatively limited growth. Calculated generation times during phase 1 (477–524 h) and 2 (>524 h) were ~ 34 h and ~ 230 h, respectively—rates comparable to those for intervals 1 and 2 of culture 1A. In growth phase 2 (Fig. 1), total cell numbers of both cultures 1A and 1B increase steadily following a power law, doubling over a fixed interval (in a pure strain, this would be equivalent to the exponential phase). Because these are mixed strain enrichments, we cannot be sure all strains are actually growing exponentially in growth phase 2. Culture 1B was removed from incubation after growth phase 2 (1256 h total).

Molecular cloning and phylogenetic inference

DNA extracted by mechanical bead-beating from culture 1A was readily amplified using both archaeal and bacterial-specific 16S rRNA gene primers, but DNA from culture 1B was only amplifiable using the bacterial primer set. The chemical extraction method yielded DNA amplifiable with archaeal primers for culture 1A only; no bacterial products were obtained from this extraction for either sample. Archaeal library 1A, yielded four nearly identical sequences

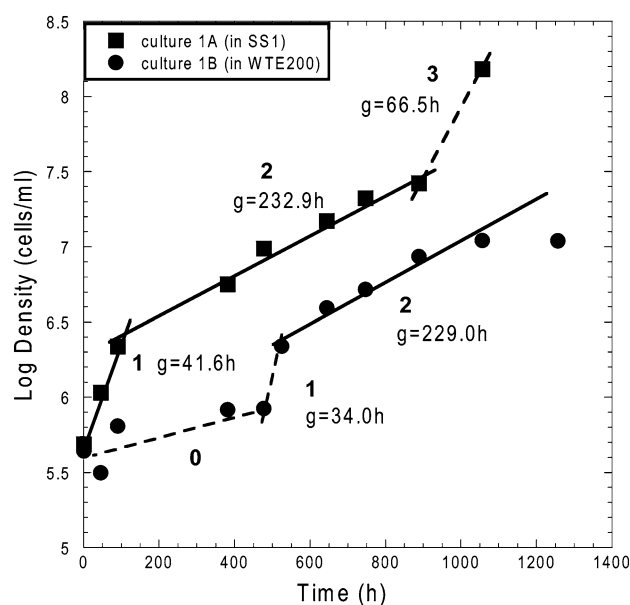


Fig. 1 Results of cell enumerations of cultures 1A (in medium SS1) and 1B (in medium WTE200) as a function of time. Numbers indicate stages of growth as discussed in the text. Dashed lines indicate growth stages proposed for poorly resolved trends or trends with few data. Cultures were grown at pH 5.5 and 80°C

(99.6% sequence similarity), which were grouped as a single operational taxonomic unit (OTU) of *Desulfurococcales* from 'Sylvan Spring' (SS1 Clone Group 1, Fig. 2). A phylogenetic tree of the crenarchaeal order *Desulfurococcales* (Fig. 2) shows the inferred phylogeny of SS1 Clone Group 1, which is most closely related to *Thermogladius shockii* (99%) and a previously published Sylvan Spring environmental clone "YNP_SSp_A84" (98%) (Meyer-Dombard et al. 2005). Cultures 1A and 1B also contained taxa whose closest homologous relatives are uncultured *gamma Proteobacteria* from environmental samples. Sequences from the bacterial clones of both cultures shared $\geq 99.5\%$ sequence similarity, and one representative clone from each was chosen for phylogenetic comparison (SS1 Clone Group 2 and WTE200 Clone Group 1 in Fig. 3). Both representatives fall in the same clade, sharing 99.2% sequence similarity. The closest cultured representatives to this OTU are *Thermomonas hydrothermalis* (AF542054) and *T. haemolytica* (AJ300185) (86% sequence similarity), and *Shewanella putrefaciens* (U91553, 81% similar).

Cell morphology

Microscopy indicated that the original 'Sylvan Spring' inoculum consisted of coccoid-shaped and rod-shaped organisms (not shown). Observations of enrichments 1A and 1B also showed cocci and rod morphologies, and a few filamentous organisms were observed (which disappeared

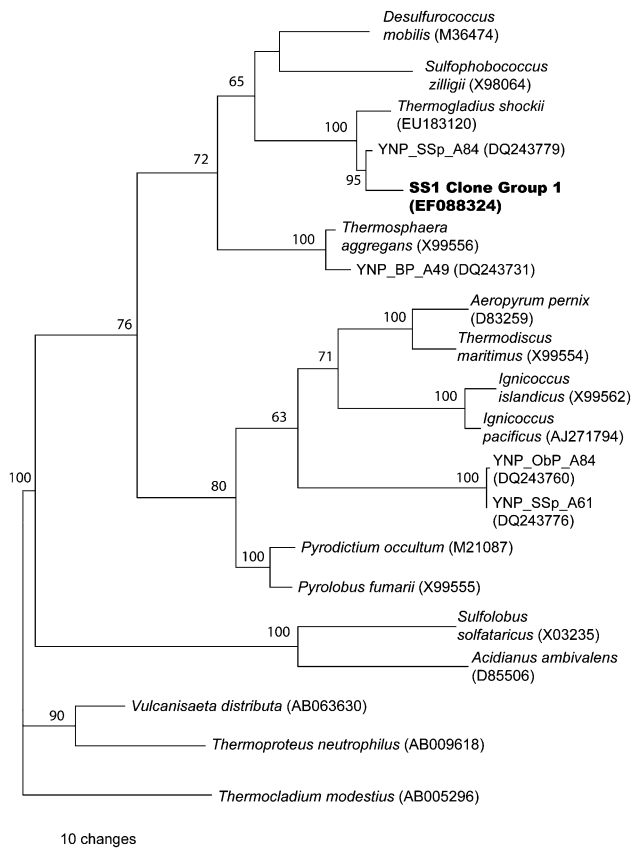


Fig. 2 Phylogenetic tree showing Crenarchaeota, by maximum parsimony analysis. The heuristic search of this dataset retrieved the tree displayed in 973/1000 replications. Longbranch attraction problems are not present, as indicated by branch lengths that are less than 10% of the total number of nucleotides aligned. 100 bootstrap repetitions. Other methods (Neighbor Joining, Maximum Likelihood) yielded similar branching topologies

from both cultures by 477 h of incubation). In growth phase one of culture 1A and putative growth phase one of culture 1B (Fig. 1), the dominant cell morphology varied. However, cocci became prevalent in 1A after 381 h of incubation and in 1B after 644 h (growth phase two in both cultures). Cell morphology at the end of the incubations agreed with that seen in TEM images, below.

TEM images from 756 h of incubation in experimental stage one (Figs. 4, 5) reveal marked differences in morphology between the communities in 1A and 1B. Images of culture 1A (Fig. 4) show regular cocci and disk-shaped cells of 1–2 μm diameter (Fig. 4a, b), and rarely, irregular morphologies (Fig. 4a, c). The majority of the cells in 1A display electron-dense cell envelopes (arrows labeled ‘2’ in Fig. 4), enlarged in Fig. 4e. Less common are cell envelopes that are gram negative with an electron ‘light’ periplasmic space (arrows labeled ‘1’ in Fig. 4a, d). Both morphologies exhibit a distinct cytoplasmic membrane (Fig. 4d, e). In comparison, cells in culture 1B are

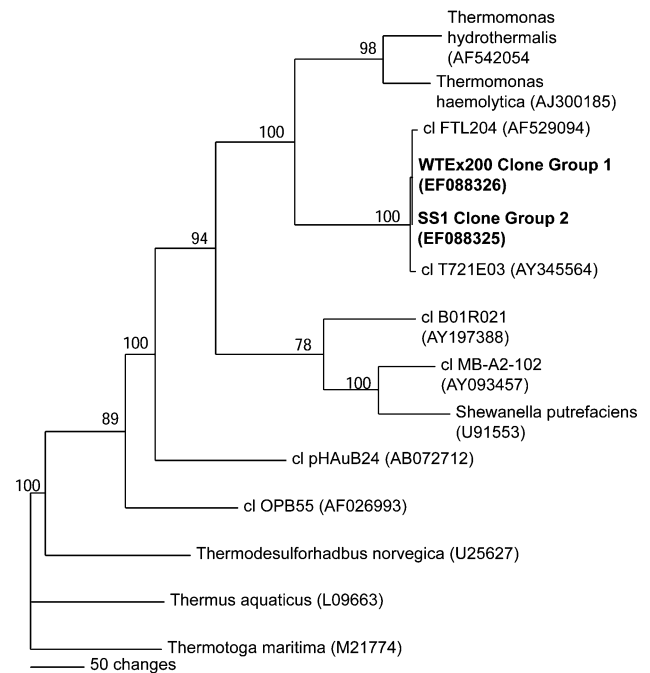


Fig. 3 Phylogenetic tree (maximum parsimony analysis) showing isolates and environmental clones of γ -Proteobacteria. The heuristic search of this dataset retrieved the tree displayed in 998/1000 replications. Longbranch attraction problems were addressed by removing individuals with branch lengths more than 10% of the total number of nucleotides aligned, and Fig. 3 displays no longbranch attraction artifacts. Numbers displayed at branch nodes are bootstrap values, out of 100 repetitions. Other methods (Neighbor Joining, Maximum Likelihood) yielded similar branching topologies

morphologically grotesque/irregular. These cells (Fig. 5a, c, d) have a thick (100–150 nm) cell envelope (enlarged in Fig. 5b) with an expanded periplasmic space. The cytoplasmic membrane is difficult to observe and the outer surface is uneven (scalloped). A common feature of cells in culture 1B is arm-like protrusions (Fig. 5c), which can also occasionally be seen in culture 1A (Fig. 4a, c). The cells of culture 1B vary from <1 to 2 μm , but the irregular cells (Fig. 5d) are much larger and of complex morphology.

Experimental stage two

In stage two, the response of the stage one cultures to changing trace element concentrations was tested and examined by epi-fluorescence microscopy and TEM. Cultures 2A (medium WTE inoculated with culture 1A) and 2C (medium SS1 inoculated with culture 1B) had cell densities at t_0 of 3.3×10^5 and 7.8×10^5 cells/mL, respectively. After 449 h, culture 2A reached a density of 3×10^7 cells/mL, and 2C a density of 1.9×10^7 cells/mL. Culture 2B (medium WTE200 inoculated with culture 1A) showed no growth even after 800 h of incubation.

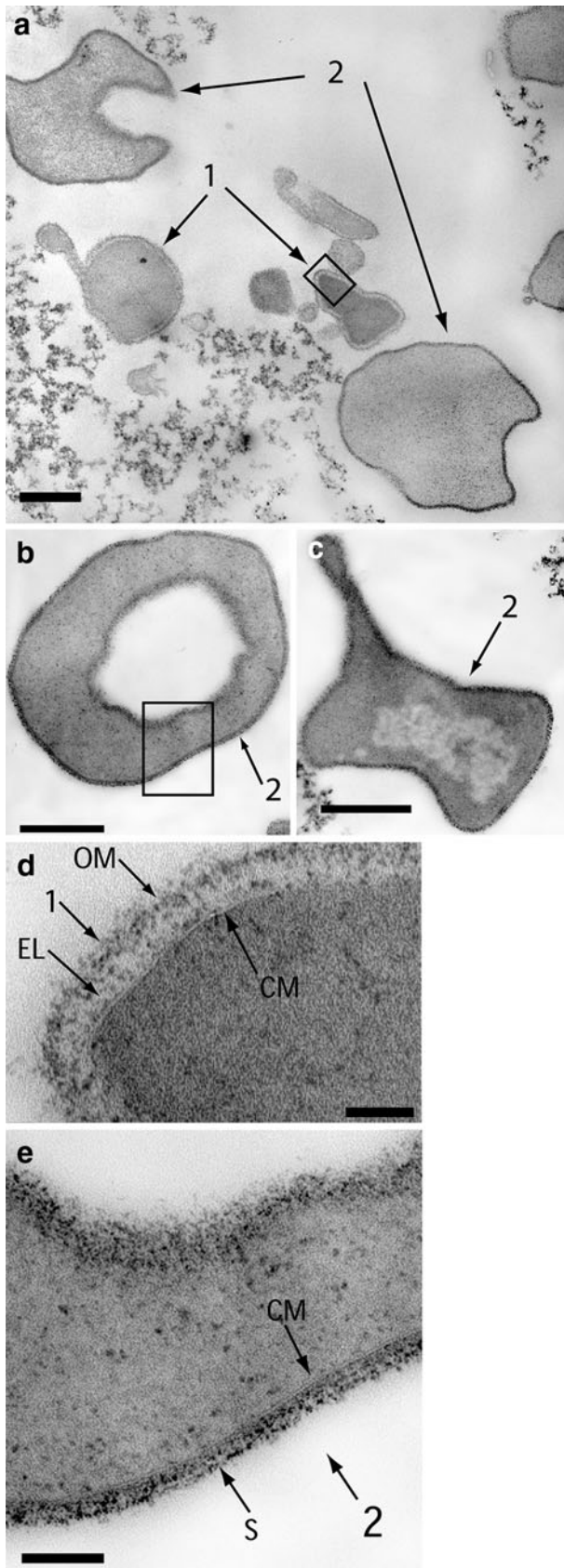


Fig. 4 TEM images of culture 1A, experimental stage one (756 h incubation). Arrows labeled as 1 indicate irregular cells with electron 'light' (EL) cell envelopes. Arrows labeled as 2 indicate disks and irregular cocci with electron-dense cell envelopes. CM cytoplasmic membrane. **a–c** Scale bars 0.5 μm . **d, e** Scale bars 60 nm. **a** Assorted cells, varying morphologies. **b** A typical disk found in culture SS1, thin section through its concavity. **c** Irregular cell, possibly dividing, displaying arm-like appendage. **d** Enlargement of cell envelope, enclosed in box in **a**, showing CM, electron 'light' periplasmic space and the outer membrane (OM). **e** Enlargement of cell envelope, enclosed in box in **b**, showing CM and S layer

TEM analysis of cultures 2A and 2C after 449 h of incubation are shown in Figs. 6 and 7. As with culture 1A, 2A consists of disk-like cells and irregularly shaped cells (Fig. 6a–c), displaying two types of cell envelopes (labeled 1 and 2) with distinct cytoplasmic membranes (Fig. 6d, e). However, culture 2A cells have different envelope morphologies than 1A cells; all envelopes are electron dense in comparison to those in culture 1A. The periplasmic space of type-1 envelopes (Fig. 4a, d) is electron 'light' when cells are grown in medium SS1 (culture 1A), but electron dense when grown in medium WTE (culture 2A) though the thickness is similar (~ 45 nm). In contrast, 1A type-2 cell envelopes (Fig. 2a–c, e) are ~ 20 nm thick, while 2A type-2 cell envelopes (Fig. 6b, c, e) are twice that (~ 40 nm) with a 20–30 nm diffuse layer external to the electron-dense layer. Evidence of an outer membrane cannot be seen in these images.

Cells in culture 2C are of comparable morphology to those in 1B (Fig. 7d), sometimes with protrusions, and 0.5–1.5 μm in diameter. However, 2C cells lack the irregular morphologies seen in the original culture 1B (Fig. 5d). Further, cell envelope structure was affected when 1B was transferred to medium SS1. While still ~ 100 nm thick, the periplasmic space is electron 'light', and has an empty appearance, lacking defining characteristics (Fig. 7b). A sharp cytoplasmic membrane is visible in 2C cells.

Discussion

Phylogenetic and ecological implications

Based on sequence similarity ($\sim 99\%$) and phylogenetic interpretation (Fig. 2), SS1 Clone Group 1 represents a close relative of *Thermogladius shockii*, of the order *Desulfurococcales* isolated from the Washburn hot spring group in Yellowstone (Osburn and Amend 2010), an organism that appears to prefer acid-sulfate type hot springs. The *Desulfurococcales* have been cultured from environments that vary in pH from 5.5 to 7, and grow optimally from 80 to 95°C—*T. shockii*'s optimum growth range is similar to the culturing conditions in this study.

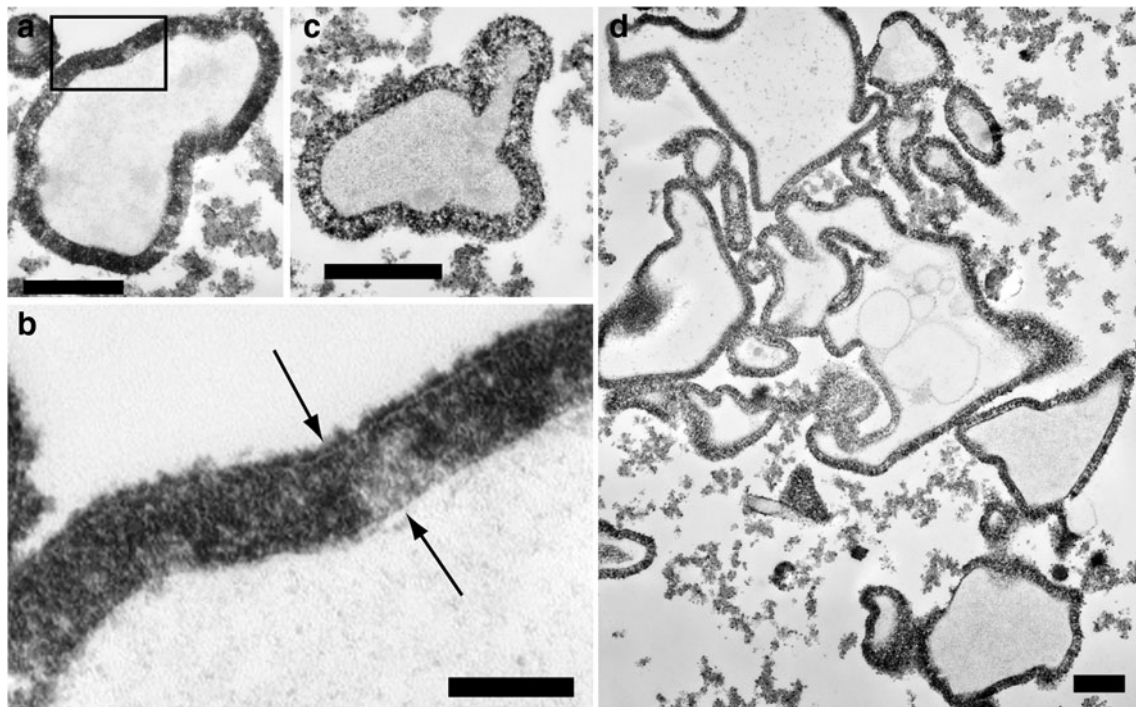


Fig. 5 TEM images of culture 1B, experimental stage one (756 h incubation). **a** Typical irregular cocci. *Scale bar* 0.5 μm . **b** Enlargement of cell envelope in **a**, shown in *box*. *Top arrow* notes scalloped membrane. *Bottom arrow* notes cytoplasmic membrane. *Scale bar*

125 nm. **c** Irregular cocci, displaying arm-like protrusion. *Scale bar* 0.5 μm . **d** View of assorted, extremely irregular ('grotesque') morphologies. *Scale bar* 1 μm

Cultured *Desulfurococcales* rely on several metabolic strategies such as organoheterotrophy, and the reduction of S^0 either with H_2 or complex organic compounds (Zillig and Stetter 1983; Bonch-Osmolovskaya et al. 2001). Surveys of community structure have found 16S rRNA signatures belonging to the *Desulfurococcales* in 'Sylvan Spring' (Meyer-Dombard 2004; Meyer-Dombard et al. 2005), and of these, clone "YNP_SSp_A84" is the most closely related to SS1 clone group 1. Clone YNP_SSp_A84 represented only $\sim 8\%$ of the archaeal clone library in the earlier study, thus culturing efforts presented here may have succeeded in capturing a "rare" strain from 'Sylvan Spring'. At a pH of 5.5, a temperature typically near 80°C , and high concentrations of H_2 and H_2S , 'Sylvan Spring' appears to be an ideal habitat for these *Desulfurococcales* (Meyer-Dombard 2004; Shock et al. 2010).

Phylogenetic inference of WTE200 Clone Group 1 and SS1 Clone Group 2 (Fig. 3) suggests that 'Sylvan Spring' may host a new clade of thermophilic *gamma Proteobacteria*. This group was also identified in a 'Sylvan Spring' sediment sample (Meyer-Dombard 2004; Meyer-Dombard et al. 2005). Other thermophilic members of the *gamma Proteobacteria* have been cultured, and are typically heterotrophic (Hallberg and Lindstrom 1994; Beeder et al. 1995; Bodrossy et al. 1999; Busse et al. 2002; Alves et al. 2003). Members of the *gamma Proteobacteria* frequently appear in 16S rRNA surveys that catalogue a wide range of

environments including hot springs, forearc basins, deep-sea hydrothermal vents, the Antarctic Dry Valleys, and gold mines (Reysenbach et al. 2000a, b; Reed et al. 2002; de la Torre et al. 2003; Dhillon et al. 2003; Elshahed et al. 2003; Ghosh et al. 2003; Inagaki et al. 2003). The geochemical range of thermophilic *gamma Proteobacteria* in extreme environments, and their apparent flexibility in the range of media tested here, points to organisms capable of adaptation to a variety of environmental stressors.

Cell growth

Differences in growth rates (Fig. 1), community phylogeny (Figs. 2, 3), and cell morphology—especially membrane structure—(Figs. 4, 5, 6, 7) document the effects of variable trace element chemistry on laboratory culturing. This study investigates the growth dynamics of mixed cultures rather than pure strains, which has implications for growth dynamics and reproducibility (Fig. 1). While a pure strain may display characteristic lag/exponential/stationary phases, the growth dynamics of mixed cultures are more complex. However, these data show that a medium with trace elements that most closely mimics the inoculum's source fluid (SS1) yields a community that grows faster and to a higher final cell density.

The trace element compositions of each medium (Table 2) vary greatly; media WTE200 and SS1 differ by

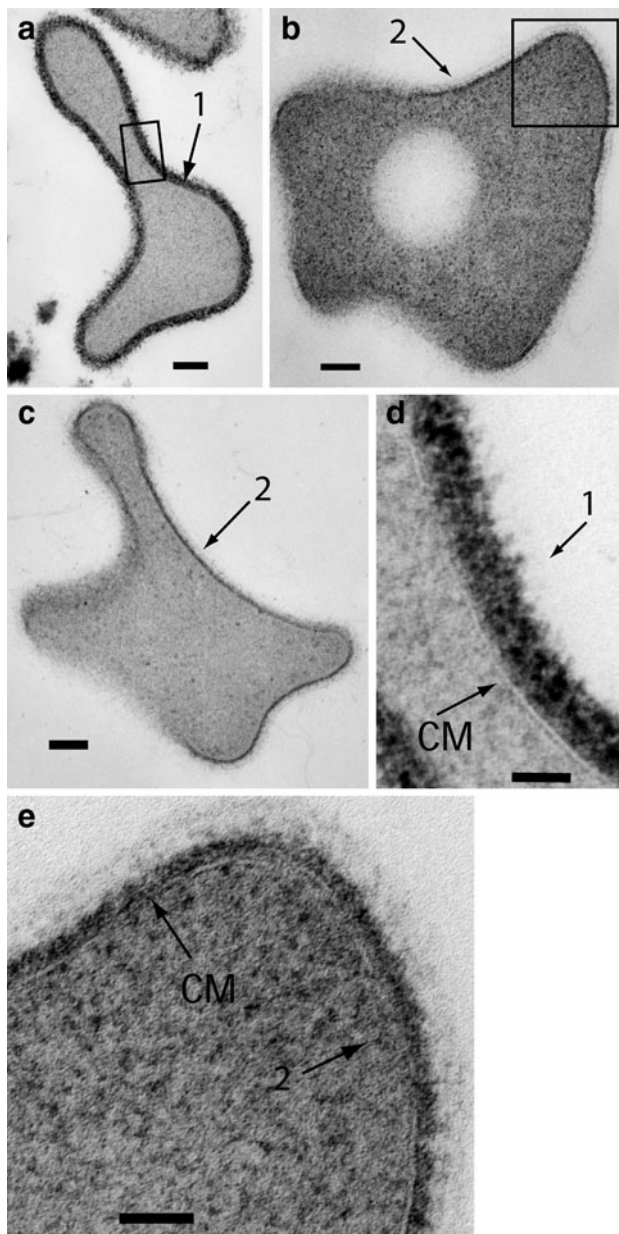


Fig. 6 TEM images of culture 2A, (culture 1A, grown in medium WTE) during experimental stage two. *Arrows* labeled as 1 and 2 indicate irregular cells with cell envelope structures comparable to those shown in Fig. 4. *CM* cytoplasmic membrane. **a–c** Scale bars 100 nm. **d** Scale bar 30 nm. **e** Scale bar 50 nm. **a** Typical cell of morphology 'type-1'. **b** Typical disk-like cell of envelope morphology 'type-2', thin section through the cell's concavity. **c** Irregular type-2 cell, as shown in Fig. 4c. **d** Enlargement of cell envelope, enclosed in box in **a**. Note lack of 'electron light' periplasmic space, as seen in Fig. 4d. **e** Enlargement of cell wall and membrane, enclosed in box in **b**. *CM* is visible, and thickening S-layer can be observed

three orders of magnitude in Fe and Zn concentrations, four orders of magnitude in Mg, Mn and Cu, and six orders of magnitude in Co. In addition, medium WTE200 did not contain salts of Rb, Sr, V, Pb, Ba, Cd and W, some of

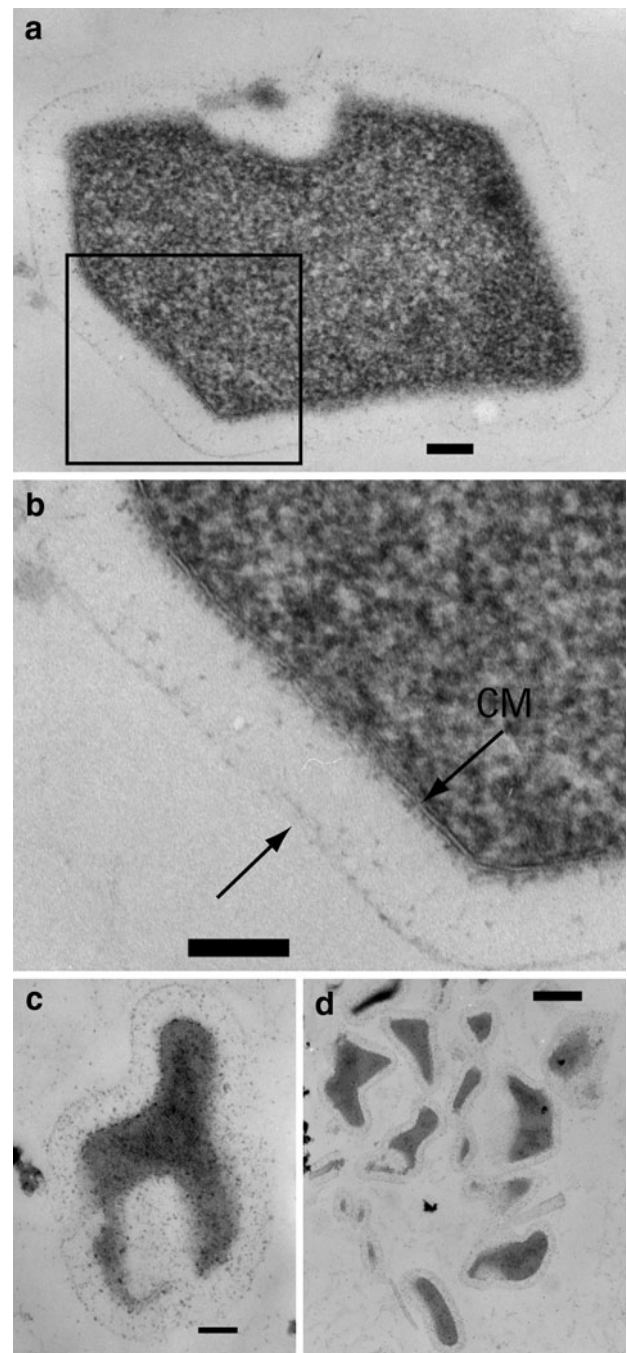


Fig. 7 TEM images of culture 2C, (culture 1B, grown in medium SS1) during experimental stage two. **a–c** Scale bars 100 nm. **d** Scale bar 0.5 μ m. **a** Typical irregular cocci, similar to Fig. 5a. **b** Enlargement of cell envelope enclosed in box in **a**. *Left arrow* notes the outer membrane. The scalloped edge seen in Fig. 5b is apparently lacking. *Right arrow* shows the cytoplasmic membrane (*CM*). **c** Irregular cell, displaying protrusion. **d** View of assorted irregular cells

which are essential to cell growth and survival (Adams 1998; Wackett et al. 2004). Addition of complex carbon substrate in the form of yeast extract contributed trace elements to the media, based on analysis of trace elements

in “ashed” dry weight yeast extract (which can be assumed to be greater on a per gram basis than the hydrated yeast extract added to the experimental media) (Grant and Pramer 1962). However, these additional trace elements were incorporated into all the experimental media, so any differences seen in these data are attributable to the additional trace elements in medium WTE200. The addition of yeast extract may contribute Co, Cu, and Mg in excess of the original SS1 trace element recipe, but not in excess of the WTE200 recipe. Based on analyses by Grant and Pramer 1962, the yeast extract contributed minimal additional Al, Ba, Fe, Mn, Mo, and Zn to the media (1–4 orders of magnitude less than recipe concentrations).

While a number of trace elements are essential, some are also known toxins (Brantley et al. 2001; Wackett et al. 2004), though our understanding of the toxin versus nutrient distinction is tempered by the inherent inability to precisely replicate complex natural systems in the laboratory. Microorganisms have developed many methods of response to environmental stress (LaPaglia and Hartzell 1997; Silver 1997; Brantley et al. 2001; Rathgeb et al. 2002). As an example, some microorganisms are known to sequester toxins within the periplasmic space between membranes in the cell wall (Silver 1997). Chemical analysis of cell membranes of the cells in 1B was not carried out in this study, however, the ultra-thick (~100 nm), electron-dense cell envelopes in the 1B Bacteria appear to result from elevated trace element concentrations, and this major morphological difference between identical species in different media may represent a physiological response to environmental stress.

Total cell numbers of culture 1A, the control for stage one of the experiment, grew quickly immediately after inoculation, but culture 1B showed little growth over the first 450 h. Complex and unresolvable data over this period (growth phase 0) may represent a sluggish community in culture 1B, or a long lag phase. The disk-shaped cells with type-2 cell envelopes (Fig. 4) are morphologically similar to members of the *Desulfurococcales*, and the type-2 cell envelope morphology (Fig. 4e) is consistent with a typical archaeal envelope, featuring an electron-dense S-layer. 16S rRNA phylogeny of cultures 1A and 1B indicate that the same bacterium grew in each medium, which is supported by the TEM images: gram-negative, type-1 irregular cells in Fig. 4 (culture 1A) are of comparable morphology to the gram-negative cells shown in Fig. 5 (1B). However, the cell envelope structures differ significantly in these two cultures, and the absence of the thick, electron-dense periplasmic space in culture 1A suggests that these morphological differences are the result of differences in medium chemistry.

The versatility and flexibility of cultures 1A and 1B were tested in experimental stage two by introducing them

into reciprocal growth media. Culture 1A was able to grow in medium WTE (culture 2A), but not in WTE200 (culture 2B). While less concentrated than medium WTE200, WTE still exceeds SS1 in Fe, Mg, Co, Cu, Zn, Mn, and Mo by 1–4 orders of magnitude, and cell envelope morphologies in culture 2A were affected by this switch in medium. The periplasmic space thickened and grew more electron dense in all cells seen. In contrast, when culture 1B was introduced to medium SS1 (culture 2C), the ultra-thick, electron-dense periplasmic space became electron ‘light’ in all cells, appearing empty and revealing the cytoplasmic membrane.

Implications for growth media design

Our second goal in this study was to incorporate our findings in the design of other growth media for various Yellowstone environments. From the ‘Sylvan Spring’ culturing experiment, several observations emerge. First, introducing cells to a trace element-rich medium containing elevated Fe, Mg, Mn, Co, Cu, Zn, and Mo induces the collection of material within the periplasmic space, in some cases widening the space significantly. Second, this process may be reversed when cells are transferred to a less concentrated medium. ‘Sylvan Spring’ derived consortia are able to grow in medium SS1 (seen in stage one of the experiment), and the mixed community resulting from this enrichment is able to acclimate to the concentrations of trace metals in medium WTE (though the cells show signs of changes to the periplasmic space, as discussed above) but not the elevated concentrations in medium WTE200. Finally, enrichments obtained by inoculating medium WTE200 did not produce as robust or as diverse a community, but these organisms were able to grow when introduced into medium SS1, showing signs of membrane alteration to more typical environmental conditions.

Trace element geochemistry in Yellowstone hot springs varies widely and is dependent on the pH, temperature, and residence time of the thermal fluids, as well as the geologic unit hosting the system. The essential elements Fe, Mg, Mn, Mo, V, as well as Sr, Pb, Ba, and W, are among the most variable—up to 4 orders of magnitude—in the locations shown in Table 1. In these experiments, better results were obtained when using a growth medium that more closely resembles the natural environment of the inoculum.

A quick comparison of trace element solutions that are part of WTE and “medium D”, commonly cited as the chosen trace element solutions for both phototrophic and chemotrophic thermophile culturing (Castenholtz 1969; Wiegel 1986; Robb et al. 1995; Boyd et al. 2007), to concentrations in natural Yellowstone hot spring samples in Table 1 shows that these traditional media differ drastically from the natural thermal environments. In

particular, the concentrations of Co, Cu, Mn, and Zn are typically far greater in these traditional media than in many Yellowstone systems. The results shown here emphasize that greater success may be obtainable by using trace element solutions that more closely resemble the natural environment.

Recipes for trace element solutions suitable for typical locations in the Lower Geyser Basin (LGB), select locations at Obsidian Pool (OBP), and select acidic locations in the ‘Sylvan Spring’ area (SS2) are also given in Table 1. These solutions were successfully employed in the design and use of growth media specific to these locations (incorporating suitable major ion chemistry for these locations, as given in Meyer-Dombard 2004; Shock et al. 2005, 2010). Culturing attempts using these and other specially designed YNP media were >70% successful in producing viable mixed cultures, and resulted in 18 isolates from the greater Obsidian Pool and ‘Sylvan Springs’ areas, and the Lower Geyser Basin (Meyer-Dombard 2004). Major ion chemistry for various YNP locations has been published elsewhere (Allen and Day 1935; Brock 1978; Ball et al. 1998, 2001; Nordstrom et al. 2005; Shock et al. 2005, 2010), and these data can be employed in the design of basal salts solutions (accounting for charge balance and the molecular weight of the salts used). In addition, data in Table 1 may be used to design conservative trace element solutions for additional Yellowstone locations. If trace element geochemistry is not available for a hydrothermal system of interest, it would not be unreasonable to take a conservative path and attempt enrichments with a diluted ‘traditional’ trace element solution, such as a diluted ‘Wolfe’s’ or ‘medium D’ trace element solutions. An alternative approach calls for using several different trace element solutions that vary in concentration.

Conclusions

Cell counts, TEM images, and phylogenetic interpretations indicate that the growth of the mixed communities in experimental stage one cultures (1A and 1B) was affected by the differences in trace element compositions. Both cultures were supplied with the same base medium (artificial ‘Sylvan Spring’ thermal fluid), metabolic constituents (complex organic compounds), inoculum (1 mL ‘Sylvan Spring’ fluid), and incubation temperature (80°C); only the trace element concentrations differed. It is concluded that medium WTE200 inhibits the efficient growth of some organisms originating from ‘Sylvan Spring’ thermal fluid, and may specifically inhibit the growth of certain *Desulfurococcales* strains from this thermal system. Further evidence that trace element concentrations affect cell growth

was seen when cultures 1A and 1B were grown in their reciprocal media.

It has been suggested that the study of extreme environments necessitates growth media that reflect the geochemical properties of the environments being studied (Baross 1995). This study further shows that trace element concentrations influence the growth of thermophiles, and may help determine the stringency of growth conditions in hydrothermal ecosystems. The results given here suggest the utility of determining the specific effects of elevated concentrations of individual trace elements, which could lead to a ranked list of elements with the strongest effects on culturing. Complementary investigations could identify which elements are stored within the periplasmic space of the cell membranes of thermophiles, and the capacity for thermophiles to sequester trace elements. Additional investigations comparing enrichments from hot springs of variable chemistry or temperature could expand on this dataset as well. Using growth media based on an environmental template may encourage the growth of species not normally culturable (such as some Crenarchaea) and combining this strategy with other recently developed culturing techniques (Zengler et al. 2002; Davis et al. 2005; Köpke et al. 2005) could expand the collection of pure isolates from extreme environments.

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