Preliminary description and nutritional characterization of a chemoorganotrophic archaeobacterium growing at temperatures of up to 110 °C isolated from a submarine hydrothermal vent environment

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An extremely thermophilic coccoid bacterium, designated strain ES4, was isolated from a flange (a newly described geological structure associated with massive sulphide mounds) at the Juan de Fuca Ridge hydrothermal vent system. The organism is a salt-requiring obligately anaerobic chemoorganotroph which reduces elemental sulphur but can grow very poorly in the absence of sulphur. Organic substrates utilized include yeast extract, peptone and amino acid hydrolysate as well as individual amino acids, the peptide Leu-Ser and starch. When growing on a defined medium, ES4 requires a supplement of vitamins and 20 amino acids at trace concentrations. Growth is most rapid in the temperature range 90–99 °C, with a maximum growth temperature of about 110 °C. In the optimal temperature range for growth, ES4 has a doubling time of about 1 h and can reach densities of 10^9 cells ml⁻¹. Insensitivity to the antibiotics vancomycin, streptomycin and chloramphenicol, and the presence of di- and tetra-ether phytanyl lipids, indicate that ES4 is an archaeobacterium.

Introduction

The discovery of submarine hydrothermal vents at seafloor spreading centres (Corliss *et al.*, 1977) revealed for the first time a biotic system in which the primary producers are aerobic and anaerobic chemolithotrophs rather than phototrophs. These primary producers in turn support a web of secondary consumers which includes a diverse population of animals and bacteria (Jannasch & Mottl, 1985). In association with this ecosystem there exist a variety of environments with a temperature range from ambient seawater temperature, about 2 °C, to greater than 400 °C (Spiess *et al.*, 1980; Delaney *et al.*, 1984). Different thermal groups of both chemolithotrophic and chemoorganotrophic bacteria have been isolated from some of these habitats (Jannasch & Mottl, 1985). Thermophilic chemoorganotrophs isolated from vent environments include members of the genera *Desulfurococcus* (Jannasch *et al.*, 1988), *Staphylothermus* (Fiala *et al.*, 1986), and *Thermococcus* (Miroschnichenko *et al.*, 1989; Neuner *et al.*, 1990) as well as strain ES1 (Pledger & Baross, 1989). All of these isolates are salt-requiring and have minimum temperature requirements for growth of 50 °C or higher and maximum growth temperatures under 100 °C. Elemental

sulphur is reduced by these heterotrophs but they vary in their degree of dependency on sulphur.

At vent sites, such as the Juan de Fuca Ridge, which lack organic sediments it is not obvious where habitats exist which combine the availability of complex organic material with a temperature range suitable for the growth of extremely thermophilic heterotrophs. One possibility is the void spaces of sulphide structures, in which animals which can be the source of exudates and decay products are often enclosed (Baross & Deming, 1985). The collapse of weathered sulphide structures burying animals and forming an insulating layer between their remains and bottom seawater and the possibility of robust chemolithotrophic communities are alternative explanations. In addition a newly described metal sulphide structure called a flange (Baross *et al.*, 1989; see Discussion) may provide a suitable habitat for extremely thermophilic heterotrophs.

In this paper we report the initial characterization of strain ES4, an extremely thermophilic coccoid archaeobacterium which was isolated from flange fragments. Strain ES4 grows at temperatures of up to 110 °C, making it the most thermophilic chemoorganotroph currently in culture. Nutritional studies with this organism have revealed that it has both a vitamin and

amino acid requirement and can be grown in a defined medium if these nutrients are supplied.

Methods

Enrichment. Rock samples from a hydrothermal flange structure were collected from the Endeavour Segment of the Juan de Fuca Ridge (47°57' N, 129°06' W) (Tivey & Johnson, 1987) by the deep-sea submersible ALVIN in August 1988. Samples were initially placed in a polyethylene bag which was flushed with nitrogen and sealed in an attempt to reduce exposure to air. Upon arrival at the University of Washington the samples were transferred to anaerobic conditions in a Gas Pak container (BBL Scientific) and stored at 2 °C.

Subsamples were taken from surface and interior regions of the flange samples and used to inoculate a series of media including the YPS medium described below. After 3 d incubation at 100 °C all tubes of YPS medium were turbid, with a mixed population of coccoid and long, thin, rod-shaped cells. Only the coccoid cells could be successfully subcultured.

Purification. To obtain a pure culture, a dilution to extinction technique (all dilutions 1:2) was employed. This process was repeated and the culture in the tube showing growth at the highest dilution was designated strain ES4. Medium and conditions were the same as reported for enrichments.

Media and growth conditions. The base for all media reported in this paper is a mineral solution (mineral base A) consisting of the following (per litre): (1) major sea salts, consisting of NaCl 19.6 g, Na₂SO₄ 3.3 g, KCl 0.5 g, KBr 0.05 g, H₃BO₃ 0.02 g, and MgCl₂·6H₂O 8.8 g; (2) 10.0 ml trace elements F, consisting of (per litre) CuSO₄·5H₂O 0.01 g, ZnSO₄·7H₂O 0.1 g, CoCl₂·6H₂O 0.005 g, MnCl₂·4H₂O 0.2 g, Na₂MoO₄·2H₂O 0.1 g, KBr 0.05 g, KI 0.05 g, H₃BO₃ 0.1 g, LiCl 0.05 g, Al₂(SO₄)₃ 0.05 g, NiCl₂·6H₂O 0.01 g, VOSO₄·2H₂O 0.005 g, H₂WO₄ 0.005 g, Na₂SeO₄ 0.005 g, NiCl₂·6H₂O 0.005 g, SrCl₂·6H₂O 0.005 g, and BaCl₂ 0.005 g; (3) 5.0 ml CaCl₂·2H₂O (1.0%, w/v, solution); and (4) 10.0 ml of a solution containing (per litre) (NH₄)₂SO₄ 43.0 g and KH₂PO₄ 3.6 g.

In addition, all media contained the buffer MES (Sigma) (1.0 g l⁻¹) and resazurin (1.0 mg l⁻¹). Unless otherwise indicated the pH was adjusted to 6.0 with 1.0 M-HCl.

YPS medium consists of mineral base A plus 1.0 g yeast extract l⁻¹, 1.0 g Bacto Peptone l⁻¹ and elemental sulphur as described below. If sulphur is omitted the medium is referred to as YP.

Vitamin solution A consists of (per litre) thiamine.HCl 50 mg, pyridoxine.HCl 100 mg, pyridoxamine.HCl 30 mg, pyridoxal 30 mg, calcium D-pantothenate 50 mg, riboflavin 20 mg, nicotinic acid 100 mg, *p*-aminobenzoic acid 10 mg, d-biotin 0.1 mg, folic acid 1 mg, cyanocobalamin 0.1 mg, and lipoic acid 5 mg.

Milli-Q deionized water (Millipore) was used for all solutions. Complete media were filter-sterilized with 0.2 µm cellulose nitrate disposable filter units or 0.2 µm cellulose acetate syringe filters (both from Nalgene). For all experiments except the one designed to test aerobic growth, air was removed and replaced with argon (ultra-high-purity – UHP – grade) by alternately applying a vacuum and saturating with gas (Balch & Wolfe, 1976). After the gas-exchange process sodium sulphide was added to a final concentration of 0.03% or 1 mM.

Unless otherwise noted all culturing was in 27 ml crimp-top tubes (Bellco) with black butyl rubber stoppers. Each tube contained 0.2 g elemental sulphur (Baker, precipitated). Sulphur was sterilized by steaming at 100 °C for 1 h on three successive days. Culturing was in either a Fisher model 129 heated water bath filled with dimethyl

silicone oil (Thomas Scientific) or Reacti-Therm heating modules (Pierce Chemical). Unless otherwise noted the temperature for growth experiments was 96 °C.

Substrates supporting growth. In an attempt to find carbon substrates which would support growth, experiments were conducted in media which were identical to YPS except that individual carbon substrates were substituted for yeast extract and peptone. These tests were run in duplicate or triplicate and a control in which no carbon substrate was added was used as a baseline since some growth was expected from nutrients added with the inoculum. Vitamin solution A (5.0 ml l⁻¹) and elemental sulphur (0.2 g per tube) were supplied unless the experiment was designed to test the ability of the organism to grow in the absence of that substrate. Glucose, sucrose, ribose, sodium acetate, sodium citrate, sodium formate and mannitol were added at a concentration of 0.2% (w/v) and sodium lactate, methanol and glycerol were supplied at 0.2% (v/v). In these experiments the temperature was 90 °C and unless otherwise noted the head space was argon (UHP grade), 100 kPa.

In other experiments, media were used which consisted of various combinations of amino acids plus mineral base A. Glycine, L-leucine, L-alanine, L-glutamic acid and L-lysine were added individually at a concentration of 20 mM and L-cysteine at 10 mM. In these experiments the complete 20 amino acids were provided at a concentration of 0.10 mM each and vitamins at 5.0 ml l⁻¹.

Another medium, referred to as '5+ amino acids medium' consists of mineral base A plus glycine, L-leucine, L-alanine, L-glutamic acid and L-lysine each at a concentration of 5 mM and the other 15 amino acids at a concentration of 0.10 mM. Other media were formulated with peptides as sole carbon and energy source. Peptides tested were Glu-Glu (α-L-glutamyl-L-glutamic acid), Ala-Pro-Gly, Glu-Lys and Leu-Ser, all at a concentration of 0.1% (w/v). Vitamins were added (5.0 ml l⁻¹) except in experiments designed to test the requirement for vitamins.

ES4 was also tested for ability to grow on yeast extract, peptone and casein enzymic hydrolysate alone and on those substrates plus vitamins. Each substrate was added to mineral base A at 0.2% (w/v). Otherwise these tests were like the single-substrate experiments described above.

Experiments similar to those with carbon substrates were conducted to test the ability of ES4 to use a range of electron acceptors. For these tests NaNO₃ was added at 0.5% and Na₂S₂O₃ and Na₂SO₃ at 0.2%. Sulphate was present in the artificial seawater used for all media.

ES4 was also tested for the ability to grow autotrophically with elemental sulphur and a headspace of H₂/CO₂ (80:20, v/v; 200 kPa), and in medium YP (i.e. without elemental sulphur). In all cases the media were anaerobic.

Determination of cell numbers and biomass. Cell numbers and biomass were determined either by direct visual counting of cells or by ATP measurement.

For direct counting, samples were filtered onto 0.2 µm polycarbonate membrane filters (Nuclepore), stained with 4',6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980), and viewed with a Zeiss UEM microscope.

For ATP determinations, samples were filtered through 0.22 µm filters (Millipore type GSWP). Filters were placed in boiling Tris buffer (pH 7.75) for 6 min, after which filter and buffer were frozen. ATP levels were determined by the firefly luciferase assay. ATP concentration has been shown to have a constant ratio to cell carbon for a wide range of organisms (Karl, 1986) and therefore is a good assay for total biomass.

Determination of the effects of temperature, salinity and pH on growth rate. All these experiments were done in duplicate except for the determination of growth rate near the upper temperature limit, where six or eight replicates were employed. Cultures were incubated without

shaking in all cases. The pH and salinity growth curves were determined at 96 °C.

When Reacti-Therm heating modules were used the wells were filled with dimethyl silicone oil in order to facilitate heat transfer and in all cases the surface of the medium in the tubes was immersed below the top surface of the heat block. Argon (UHP) was used as the head-space gas at a pressure of 100 kPa (200 kPa for experiments at 108 °C or higher) at the beginning of each experiment and was renewed as required in order to maintain an internal pressure sufficient to prevent boiling. Cultures were sampled periodically and fixed with glutaraldehyde at a final concentration of approximately 2% (v/v).

For the determination of growth rates at different pH values, modifications of YPS medium were used with the following buffers, each at 1.0 g l⁻¹: (1) pH 3 and pH 4, no buffer; (2) pH 5 and pH 6, MES; (3) pH 7, PIPES; (4) pH 8, HEPES; (5) pH 9, no buffer. All buffers were obtained from Sigma. After addition of the Na₂S the pH was restored to the appropriate value by the addition of a predetermined quantity of 1 M-HCl.

For the determination of growth rates with varying salt concentrations a three times normal concentration of the major sea salts described under 'Media and growth conditions' was prepared and diluted as appropriate with deionized water. These diluted salt solutions were used to prepare mineral base A and YPS medium.

Temperature monitoring. Temperature was monitored in the Reacti-therm heat modules with ASTM model 95C or 94C thermometers, which have a precision of 0.1 °C. Thermometers were inserted in the thermometer wells of the heat blocks. Temperature in the Fisher water bath was monitored with the installed thermometer, which was checked against an ASTM thermometer and found to give the same readings within 1 °C. In order to further evaluate the actual temperatures experienced by cultures, measurements were performed with thermometers inserted directly into tubes of medium through the rubber stoppers and with chromel/alumel (model 5TC-TT-K-20-36) precision thermocouples from Omega Engineering. It was determined that actual temperatures experienced by cultures were approximately 4 °C lower than those measured by the external thermometers, presumably due to evaporative cooling.

Sulphide generation. Cultures in YPS medium with an argon (UHP, 100 kPa) head space were monitored for production of H₂S. Uninoculated controls were run in parallel. All cultures were run in triplicate. Samples were preserved by combining with excess ZnSO₄·7H₂O in 1 M-NaOH (Ingvorsen & Jørgensen, 1979). The method of Cline (1969) was used for all sulphide measurements.

Electron microscopy. Cells were cultured in 1 litre of YPS medium, harvested in the late exponential growth phase and preserved in 2.5% (v/v) glutaraldehyde. The pellet was resuspended in 0.2 M-cacodylate buffer at pH 6.8, post-fixed with 1% (w/v) OsO₄, thin-sectioned, stained with 1% (w/v) uranyl acetate, and viewed with a Philips model 300 transmission electron microscope.

Lipid analysis. Lyophilized cell material was extracted by the method of Bligh & Dyer (1959) as modified by White *et al.* (1979). Archaeobacterial ether lipids were released by strong acid methanolysis (Gehron & White, 1983) and extracted into hexane/chloroform (1:1, v/v). Ether-linked lipids were quantified by supercritical fluid chromatography (Deluca *et al.*, 1986).

Antibiotic sensitivity. Sensitivity to the antibiotics vancomycin, streptomycin and chloramphenicol (all from Sigma) was tested at a concentration of 100 p.p.m. The temperature for this test was 70 °C. Previous experiments with the eubacterium *Thermus aquaticus* (ATCC 25104) have indicated that the antibiotics are effective at the test temperature.

DNA base composition. Cells were cultured in approximately 3 litres of YPS medium at 80 °C and harvested in the late exponential phase of growth. The procedures for processing of cells and purification of DNA were identical to those described by Pledger & Baross (1989).

Results

Morphology and ultrastructure

Cells of ES4 are coccoid and typically slightly irregular (Fig. 1*a*). Cell diameters vary from about 0.8 to 1.2 µm. No flagella or pili have been seen. Gram stains of ES4 have yielded variable results.

Transmission electron micrographs of ultra-thin sections (Fig. 1*b, c*) reveal an electron-dense bi-layered membrane surrounded by a cell wall. The outer surface shows evidence of a paracrystalline protein surface layer (Koval, 1988).

Nutrition

ES4 is an obligately anaerobic elemental sulphur-reducing chemoorganotroph. Autotrophic growth was not detected after 24 h on a mineral medium plus H₂, CO₂, vitamins and elemental sulphur, and no aerobic growth on YP medium (containing no sulphur) was detected. Nitrate, sulphate, sulphite and thiosulphate did not substitute for elemental sulphur as an electron acceptor. Very weak growth was detected after a lag of about 12 h on YP medium incubated anaerobically. Final cell density was below 10⁷ cells ml⁻¹.

On complex organic media such as YPS, ES4 grows vigorously with a generation time of about 1 h (specific growth rate = 0.67), reaching final cell densities of greater than 10⁹ cells ml⁻¹ under ideal conditions. Similar growth rates with somewhat lower final cell yields were obtained with yeast extract and peptone alone, whereas with casein hydrolysate as sole organic substrate growth rates and yields were lower (Table 1). It is interesting that ES4 would show growth of up to three doublings and reach densities of approximately 10⁷ cells ml⁻¹ in the absence of the primary carbon source in these experiments. Fig. 2 shows this result in a peptone medium and is typical of similar experiments in casein hydrolysate and yeast extract. Addition of vitamins resulted in enhanced growth rates and yields which were two to five times higher.

Experiments were conducted in an attempt to determine a defined medium for ES4 (Table 1). When provided with 20 amino acids (each 0.10 mM) and vitamins, ES4 grew on L-glutamic acid, L-leucine, glycine and starch. No growth was seen with L-alanine, L-cysteine, L-lysine or any other individual substrates

Fig. 1. (a) Electron micrograph of ES4 grown in YPS medium at 80 °C. Platinum shadow, showing a fairly regular-sized coccoid morphology typical of exponential-phase cultures. (b, c) Electron micrographs of ultrathin sections of ES4 grown in YPS medium at 80 °C.

Table 1. Maximum cell yields and growth rates for ES4 with various carbon-containing substrates

All tests were conducted at 90 °C. All media included elemental sulphur.

Medium	Max. cell density (cells ml ⁻¹)	Specific growth rate (h ⁻¹)	Concn of C-containing substrate in medium (mg ml ⁻¹)	Max. cell density per mg C-containing substrate (cells mg ⁻¹)
YPS (0.1)*	1 × 10 ⁸	0.67	2.0	5 × 10 ⁷
YPS (0.5)*	3 × 10 ⁸	0.70	10.0	3 × 10 ⁷
Yeast extract†	1 × 10 ⁸	0.64	2.0	5 × 10 ⁷
Peptone†	2 × 10 ⁸	0.51	2.0	1 × 10 ⁸
Casein hydrolysate†	8 × 10 ⁷	0.28	2.0	2 × 10 ⁷
Yeast extract, trace‡	7 × 10 ⁷	0.51	0.4	2 × 10 ⁸
'5+ amino acids' plus vitamins§	3 × 10 ⁷	0.10	3.3	1 × 10 ⁷
'5+ amino acids' plus cyanocobalamin§	5 × 10 ⁶	0.15	3.3	2 × 10 ⁶
20 amino acids (2 mM) plus vitamins	1 × 10 ⁷	0.31	5.8	2 × 10 ⁶
Leu-Ser (0.1%) plus vitamins	4 × 10 ⁶	0.19	1.0	4 × 10 ⁶
Selected individual carbon substrates				
L-Glutamic acid	6 × 10 ⁷	0.12	3.2	1 × 10 ⁷
Glycine	4 × 10 ⁷	0.23	1.8	2 × 10 ⁷
L-Leucine	2 × 10 ⁷	0.12	3.0	1 × 10 ⁷
Starch	4 × 10 ⁷	0.14	5.0	1 × 10 ⁷

* Number in parentheses indicates the concentration (% w/v) of both yeast extract and peptone.

† Identical to YPS except the indicated substrate is present at 0.2% and is the only carbon-containing substrate.

All media contain vitamins.

‡ Identical to YPS except peptone is omitted and yeast extract is added at a concentration of 0.04%. This is the lowest concentration of the substrate which resulted in detectable growth.

§ See Methods, 'Substrates supporting growth'.

|| Growth was not detected on the other peptides tested (Glu-Glu, Ala-Pro-Gly, Glu-Lys).

tested. Growth did not occur on starch or any subset of the amino acids without vitamins or without the 20 amino acids at trace levels. Ongoing experiments with ¹⁴C-labelled substrates show that ES4 can incorporate amino acids and acetate but not glucose.

Growth was obtained in '5+ amino acids medium' when complete vitamins (Fig. 3) or cyanocobalamin alone were present, but growth yields were lower when only cyanocobalamin was present. No other individual vitamin was sufficient to allow growth in that medium.

Very weak growth, approximately a 10-fold increase in cell number, was detected on the peptide Leu-Ser but no growth was detected with the other peptides tested. In comparison with growth on rich media, growth on defined media was poor in all cases, with generation times of 3 to 7 h, and final cell yields per unit of carbon-containing substrate were often an order of magnitude or more lower than on YPS (Table 1).

Effects of pH and salt concentration

Growth was observed over a pH range of 4.0–8.0, with optimal growth occurring over the range 5.0–7.0. No growth was observed at pH 3.0 or 9.0. The reported pH values were measured at the beginning of each experi-

ment. Metabolic products caused the pH to rise over the course of these experiments. In cultures with an initial pH of 4.0 or 5.0, the pH increased by about 1 pH unit in 9 h. The increase was less in higher-pH cultures, due to buffering.

ES4 grew over a rather narrow range of total sea salts, showing no growth when salts were diluted to $0.3 \times S$ ($S = 32.3 \text{ g total salts l}^{-1}$) or increased to $2.0 \times S$.

Temperature range for growth and behaviour at high temperatures

ES4 grew over a temperature range of about 66–110 °C (Fig. 4). Most rapid growth occurred between 90 and 99 °C, with a generation time of about 1 h. Growth at temperatures above 102 °C was repeatedly observed but erratic in pattern, with final cell densities generally below $10^7 \text{ cells ml}^{-1}$. Replicate cultures frequently differed widely in growth rate, with some replicates not showing growth (Fig. 5a, b). Cultures often showed an initial decline followed by recovery. Similar results were obtained whether growth was monitored by ATP assay or direct counts. The complex behaviour of ES4 cultures at high temperatures is the subject of continuing investigation in our laboratory (see Discussion).

Fig. 2. Growth of ES4 with peptone (0.2%, w/v): O, plus vitamins; ●, without vitamins. □, Control without carbon substrate (peptone).

Fig. 3. Growth of ES4 in '5-plus amino acids' medium: O, plus vitamins; ●, without vitamins. At 8 h cell numbers were below the threshold of detection (about 1×10^5 cells ml⁻¹) in the culture without vitamins (*).

Fig. 4. Specific growth rates of ES4 at various temperatures within its growth range in YPS medium at pH 6.0.

Fig. 5. Growth of ES4 at (a) 108 °C and (b) 110 °C as determined by increases in ATP concentration with time in YPS medium at pH 6.0. Each line represents a replicate growth experiment.

Fig. 6. Generation of H₂S and cell number increase of ES4 grown in YPS medium and incubated at 96 °C. ■, H₂S generation in uninoculated YPS medium with an argon head space; ●, H₂S generation in inoculated YPS medium with an argon head space; □, cell number increase in YPS medium.

Sulphide generation

Growth of ES4 on YPS medium with a gas head space of argon was accompanied by the copious generation of H₂S (Fig. 6). Abiotic production of H₂S from elemental sulphur was also observed (Belkin *et al.*, 1985) in uninoculated controls, though at a trivial rate in comparison to biological production.

Antibiotic sensitivity

ES4 was insensitive to the antibiotics chloramphenicol, vancomycin and streptomycin at a concentration of 100 p.p.m.

Lipid composition

Archaeobacterial ether lipids were present at 28 mol% diether and 72 mol% tetraether form. No significant quantities of fatty acids were detected. The tetraether did not show any of the cyclization characteristic of *Thermoplasma* sp.

DNA base composition

The G + C content was determined as 55.0 mol%.

Discussion

Several reports have indicated that coccoid, chemoorganotrophic, salt-requiring archaeobacteria are a prominent component of the bacterial population at submarine hydrothermal regions. Our experiences support this conclusion and at present we have in culture over 20 strains of thermophilic chemoorganotrophs from various vent environments. The isolate described in this paper, ES4, from the Endeavour Segment of the Juan de Fuca Ridge, grows at temperatures of up to 110 °C. Strain ES4 most closely resembles strain ES1 (Pledger & Baross, 1989), *Hyperthermus butylicus* (Zillig *et al.*, 1990), and *Desulfurococcus* (Jannasch *et al.* 1988). However, ES4 differs from ES1 by its upper temperature for growth and dependency on elemental sulphur, and from *Hyperthermus* and *Desulfurococcus* by several metabolic features including growth on a mixture of 20 amino acids. Other differences from previously described coccoid thermophilic archaeobacteria from submarine vent regions include the mol% G + C value, degree of dependency on elemental sulphur, presence or lack of flagellation, and cell morphology.

This paper constitutes the fourth report of an axenic strain growing in the laboratory in the temperature range 105–110 °C (Stetter *et al.*, 1983; Huber *et al.*, 1989; Zillig

et al., 1990). All studies so far with these organisms and with ES4 have been conducted at a hydrostatic pressure at or near 1 atm. Since ES4 was isolated from a depth of about 2200 m (22.3 MPa) it is possible that hydrostatic pressure may have an influence on its temperature range for growth and survival as well as on various metabolic processes (Morita, 1972; Jaenicke, 1983; Cowan, 1989). It has also been proposed that pressure may extend upward the maximum temperature at which organisms can grow (Baross *et al.*, 1984). Although this is clearly not the case for all organisms (Bernhardt *et al.*, 1988; Miller *et al.*, 1988), too few data exist at present to resolve the issue.

The behaviour of ES4 in the temperature range of 102 °C to its maximum temperature for growth of about 110 °C is suggestive of a complex physiological adaptive response similar in nature to the heat-shock response which involves the synthesis of a distinct set of proteins (Neidhardt & VanBogelen, 1987). The heat-shock response has been observed in eubacteria and eukaryotes and in the extreme halophiles (Daniels *et al.*, 1984) and *Sulfolobus* sp. (Jerez, 1988; Trent *et al.*, 1990) among the archaeobacteria but has not been investigated in extremely thermophilic archaeobacteria isolated from deep sea vent environments. Since organisms from these environments are likely to experience wide variations in key environmental parameters such as temperature, pH, *E_h* and exposure to oxygen, we believe that they are an ideal group in which to investigate the ecological and physiological aspects of the heat-shock response.

Defining the nutritional requirements of ES4 was given priority in this study because of the insights that this might provide into the nature of the organism's habitat and because of our interest in further physiological and genetic investigations. The '5+ amino acids' medium reported in this paper constitutes a defined medium although more work will be necessary to overcome the limitations imposed by poor cell yields. Possible reasons for these low yields on amino acids as compared to the yields on complex substrates include utilization of only a small number of the amino acids or limitation by some as yet unidentified nutrient provided in trace amount with other ingredients. The latter explanation seems more likely since the yield per unit of carbon-containing substrate did not change drastically with variation in the amino acids supplied (Table 1). It is also possible that utilization of some required nutrient is enhanced by the presence of chelating factors or other substances present in the complex media.

This is the first report of an extremely thermophilic sulphur-reducing chemoorganotroph which requires vitamins and amino acids for growth in a defined medium. That the organism has such a complex nutritional requirement has important implications for

understanding the ecology of ES4 and other chemo-organotrophic isolates from volcanic environments. ES4 was cultured from a newly described geological structure called a flange (Baross *et al.*, 1989). A flange is a horizontal outcropping from a metal sulphide mound beneath which hot buoyant hydrothermal fluids form inverted ponds before spilling over the edge and rising into the water column. The upper surface of a flange is cool enough to support a dense animal population from which exudates and decay products may find their way into the interior of the flange by burial or diffusion. Although the processes of formation of flanges and the details of their structure are still largely unknown there is reason to believe that the temperature and chemical gradients may be relatively stable (Delaney *et al.*, 1988). This stability, combined with the relative ease of access, makes the flange a suitable environment for investigating the ecology of extremely thermophilic organisms and for addressing such questions as the upper temperature for growth and survival of micro-organisms.

The study of the physiology and biochemistry of extremely thermophilic archaeobacteria is still in its infancy. Studies in this field have often been hampered by the lack of defined media and the tendency of cultures to cease growth at low final cell densities. Since these problems have been partially solved with strain ES4, and because of its exceptionally thermophilic nature, we propose that ES4 is a favourable organism with which to address physiological, biochemical and biotechnological questions pertaining to extreme thermophily.

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