New Zealand Journal of Marine and Freshwater Research, 1986, Vol. 20: 439–445
0028–8330/86/2003–0439$2.50/0

Isolation of anaerobic, extremely thermophilic, sulphur metabolising archaebacteria from New Zealand hot springs

B. K. C. PATEL[1]
P. M. JASPERSE-HERST
H. W. MORGAN
R. M. DANIEL
Department of Biological Sciences
University of Waikato
Private Bag, Hamilton
New Zealand

Abstract Enrichments of New Zealand geothermal samples, initiated in anaerobic sulphur-containing media and incubated at temperatures above 85°C, yielded rod and coccal shaped organisms which possessed archaebacterial characteristics. Pure cultures were isolated and characterised. Five of the seven isolates, which were rod-shaped organisms and did not have an obligate requirement for sulphur respiration, were similar to *Thermoproteus* sp. but had more neutral pH optima for growth. Three of these five *Thermoproteus* sp. were obligate heterotrophs, which has not previously been reported. The two coccal isolates had an obligate requirement for sulphur as an electron acceptor and were similar to *Desulfurococcus* sp. but again with more neutral pH optima for growth.

Keywords hot springs; archaebacteria; *Thermoproteus*; *Desulfurococcus*; sulphur respiration; geothermal; growth; optima

INTRODUCTION

Interest in the microbiology of extreme environments, especially the geothermal environment from which extremely thermophilic anaerobic archaebacteria have been isolated, has been increasing for the past decade. Archaebacteria, which form the third Urkingdom of organisms (Fox et al. 1980), have provided a different perspective on the evolution of life and hence are of interest to evolutionary biologists. The possibility that these organisms, which have optimum growth temperatures between 80 and 105°C (Fischer et al. 1983; Stetter et al. 1983), can be exploited to develop novel biotechnological processes, e.g., production of thermostable enzymes (Buonocore et al. 1980), has interested industrial microbiologists and biotechnologists.

A number of extremely thermophilic anaerobic archaebacteria with growth optima between 85 and 100°C have been described. These include *Thermoproteus* sp. (Zillig et al. 1981; Fischer et al. 1983), *Desulfurococcus* sp. (Zillig et al. 1982), *Thermofilum pendens* (Zillig et al. 1983a), *Pyrodictium* sp. (Stetter et al. 1983), *Thermococcus celer* (Zillig et al. 1983b), and the methanogenic *Methanothermus* sp. (Stetter et al. 1981).

All of these organisms have been isolated from thermal areas of the Northern Hemisphere (Italy, Iceland, Japan, and USA) and have not been reported from the thermal areas of the Southern Hemisphere. Geography appears to be an important factor in their distribution since *Thermoproteus* sp. and *Desulfurococcus* sp. could not be isolated from a number of springs in Italy and Japan (Zillig et al. 1981, 1982). *Pyrodictium* sp. and *Thermococcus celer* were isolated from Italian but not from Icelandic springs (Zillig et al. 1983b). Studies of the thermal springs of the Southern Hemisphere, especially those of New Zealand which have a higher boiling point and generally higher sulphide content than most thermal springs from other parts of the world (Brock 1978), might reveal novel types of archaebacteria. We report here the ecology and physiology of some archaebacteria found in New Zealand thermal springs.

Received 28 November 1985; accepted 3 February 1986

[1]Present address: Ruakura Animal Research Station, Ministry of Agriculture and Fisheries, Private Bag, Hamilton, New Zealand.

Table 1 Results of enrichment of bacteria in PEM at an incubation temperature of 92°C.

Pool no.	Temp. (°C)	pH	Bacterial morphotype Rods to filaments	Cocci
ROTORUA				
Rt3* (602)	70	7.2	-	-
Rt8*	64	9.1	-	-
Rt12 (428)	82	6.9	+	+
Rt13* (364)	97.5	6.9	+	-
Rt14* (358)	82	7.2	+	+
Rt15* (351)	96–102	8.7	+	+
Rt30*	97	3.69	-	-
Rt41 (360)	100	7.4	+	-
Rt42*	97	7.3	+	+
Rt51* (426)	82	7.3	+	+
Rt56*	90	7.9	+	+
Rt57*	97	7.2	+	+
Rt58 (711)	103	8.1	+	-
Rt59 (720)	103	7.2	+	+
Rt60 (715)	97.7	7.2	+	+
Rt61	94.5	8.1	+	-
Rt68	95	7.5	+	+
Rt70	98.2	3.4	-	-
Rt71	98.5	3.3	+	+
Rt72	97	6.6	+	+
Rt74	NA	NA	+	+
Rt80	NA	NA	+	+
Rt82	91	6.76	+	+
Rt84	NA	NA	+	+
Rt87 (396)	94	7.8	+	+
Rt88 (392)	95	8.3	+	+
Rt89 (388)	94	8.2	+	+
Rt90 (386)	98	7.3	+	+
Rt91 (383)	96	7.6	+	+
Rt93	96	6.8	+	+
Rt98 (354)	100	8.5	+	+
Rt99 (363)	98	4.8	+	-
WAIMANGU				
Wai8	85	3.03	-	-
Wai9	88	3.1	-	+
Wai11	96	8.5	+	+
Wai17	80	8.5	+	-
Wai18	81	7.34	-	+
Wai19	79	7.7	-	-
Wai21	67	6.5	-	-
WAIRAKEI				
Wk1	97	3.15	-	-
WAIKETI VALLEY				
Wkv6	96	6.5	+	+
KETATAHI				
Ket10	89	6.7	+	+
TOKAANU				
Tok12	97	6.9	+	+
TAUPO				
Tp44	99	7.1	+	+

NA = Not analysed. Media used was as described in Materials and Methods. An increase in cell numbers and sulphur corrosion was used as an index of growth.

*Indicates that enrichments were performed at least twice at different times and identical results were obtained. Official DSIR numbers of the pools (wherever available) have been listed in parenthesis.

METHODS

Enrichment and isolation

Pools covering the temperature range from 64 to 103°C and a pH range from 3.1 to 9.1 were sampled from a number of thermal regions of New Zealand (Table 1). Procedures for sampling, pH, and temperature measurement and for the preparation of anaerobic media were performed as previously described (Patel et al. 1985a, 1985b, 1985c).

Initial enrichments were performed in an anaerobic pool enrichment medium (PEM). PEM consisted of pool water as a basal medium to which nutrient sources were added. An inoculum source of 9 ml pool water and 1 ml sediment slurry was added to sterile tubes. Other additions were made to the tubes from sterile stock solutions: 0.1 ml of 1% trypticase peptone-yeast extract mixture, and 100 µl of 10% $Na_2S.9H_2O$. Twenty milligrams of sulphur (non-sterilised) and resazurin were also added to each tube. Air in the head space was replaced with nitrogen by flushing with oxygen-free nitrogen and the tube sealed with a Hungate cap. Because of the reducing nature of the thermal sediments and the addition of sulphide, anaerobic conditions quickly prevailed as evidenced by resazurin reduction. Replicate enrichments from each site were incubated at 85, 92, and 95°C. Control tubes were prepared similarly and in addition contained 0.4 ml of 40% formalin. These acted as controls for determination of initial cell counts present before enrichments. To determine cell growth in enrichments, initial and final cell numbers were determined using a Petroff-Hausser cell counting chamber (C. A. Hausser and Son, USA) as described by the manufacturer.

Db medium was essentially as described by Zillig et al. (1981) except that the pH of the medium was adjusted to 6.8. It was also used to initiate enrichments and for all subsequent transfers of cultures.

Isolation of pure cultures from enrichments was not possible using conventional procedures involving agar because at the temperature of incubation agars did not remain sufficiently gelled. For the rods, pure cultures were obtained by repeated transfers at 98°C until no cocci appeared when reinoculated at 88°C. The culture was then taken through at least two successive end-point serial dilutions. Enrich-

Table 2 Characteristics of *Thermoproteus* and *Desulfurococcus* species. ND = not determined.

Strain	Growth range Temp (°C)	pH	Growth optima Temp (°C)	pH	Generation time (h)	Auto-trophy	Growth on casein
COCCI							
Rt59-S1	65–95	4.0–8.0	88	6.8	4.0	No	+
Tok12-S1	65–95	4.0–7.5	88	6.0	3.25	No	+
Desulfurococcus mobilis	ND	4.5–7.0	85	6.0	3.0	No	+
Desulfurococcus mucosus	ND	4.5–7.0	85	6.0	4.0	No	ND
RODS							
Rt13-S1	75–98	4.5–7.5	90	6.0	5.5	No	–
Tok12-S2	75–98	4.5–7.5	90	6.5	8.0	No	–
Ket10-S1	75–98	4.5–8.0	90	6.5	13.5	No	–
Wkv6-S1	70–98	4.5–8.0	88	6.4	6.5	Facultative	–
Tp44-S1	82–98	5.0–8.0	90	6.9	6.0	Facultative	–
Thermoproteus tenax	ND	ND	88	5.5	1.7	Facultative	–
Thermoproteus neutrophilus	ND	ND	85	6.8	4–8	Obligate	–

In addition, *D. mobilis* but not *D. mucosus* is motile and *D. mucosus* but not *D. mobilis* has a slimy polymer attached to its envelope.

ments with cocci were freed of contaminating rods by successive transfer at 70°C on Db medium containing casein instead of peptone. When no rods were present on subsequent incubations at 85°C on normal Db medium, the culture was taken through at least two successive end-point serial dilutions. The pure cultures so obtained were stored by freeze drying.

The numbers of organisms present in pools were estimated from sediment slurry or pool water as an inoculum source. The preparation of the sediment slurry for enumeration was as follows: Sediment as well as pool water was collected and the sediment allowed to settle out for six hours, after which the supernatant was siphoned out and a 1:1 dilution of the sediment prepared using the appropriate medium. A twenty fold dilution was made by adding 1 ml of the sediment slurry to 9 ml of medium, from which serial dilutions in Db medium were prepared. When pool water was used, serial dilutions were prepared directly without the twenty fold dilution step. Triplicate tubes were inoculated from each dilution and incubated at 88 and 95°C for determination of bacterial numbers by the MPN method (APHA 1973).

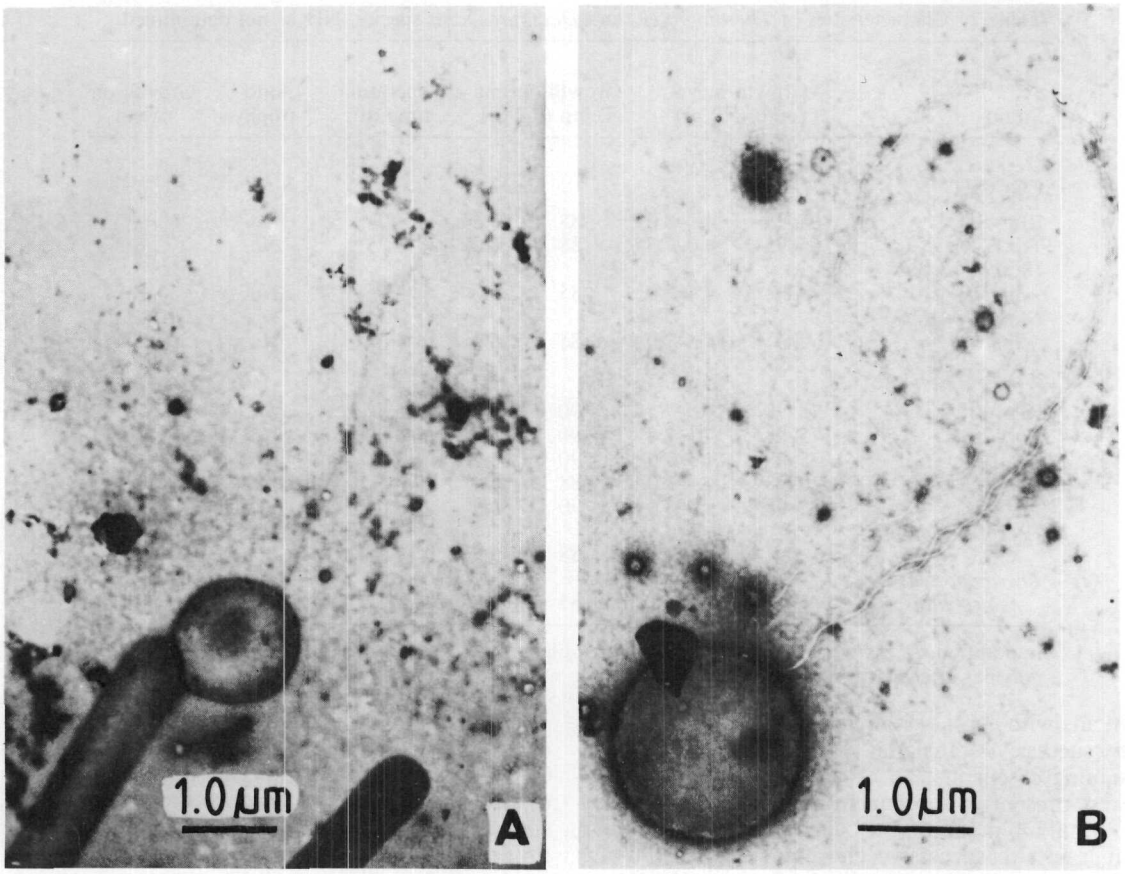
Growth and cellular characterisation

Electron microscopy and phase contrast microscopy were performed for cellular characterisation as described previously (Patel et al. 1985a, 1985b, 1986).

For nutrient utilisation tests, the yeast extract concentration of Db medium was reduced to 0.0001% which was insufficient to sustain growth of any cultures tested. Carbon sources were added singly from filter sterilised stocks to a concentration of 0.2% or in combination with 0.02% yeast extract. Either sulphur or 0.1% cystine was used as electron acceptor. Sulphur was not sterilised before use, and no growth was observed in uninoculated control tubes which contained unsterilised sulphur. Tubes were inoculated with 0.1 ml of inoculum to avoid carry over of nutrients and incubated at 90°C. After three days tubes were examined microscopically for growth. Cell numbers of tubes exhibiting growth were counted using a Coulter Counter (Model ZB1) with a lower threshold of 5, an aperture current of 1 mA, an amplitude of 1/2 (cocci) or 1/4 (rods), and a counting tube of 30 μm orifice. Mean values were corrected with the appropriate coincidence factor [(mean value/1000)^2 × 0.3375]. Where growth was observed the tubes were used as an inoculum into the same medium to confirm the growth and cell density obtained.

Table 3 Most probable numbers of *Thermoproteus* type cells in pool Rt13.

Inoculum source	No. of cells ml^{-1} 88°C	95°C
Pool water	1.1×10^3	4.6×10^2
Sediment slurry	4.3×10^4	9.3×10^3



RESULTS AND DISCUSSION

In general, enrichments initiated in both media gave rise to the same mixed bacterial population of rods, filaments, and cocci at 85 and 95°C, whereas only rods were present in enrichments at 98°C with the exception of pool Rt42 where a coccus associated with the rods was also present. The cell wall structure of the coccus in pool Rt42 differed from the cocci enriched from other pools at lower incubation temperatures and was very similar to that of a rod present in the pool and with which it was frequently found in association (Fig. 1). A pure culture of the coccus was not obtained, and it was not investigated further. Such cocci-rod associations have been reported previously (Zillig et al. 1981).

Positive enrichments were obtained from 36 out of the 44 pools tested (Table 1). Of the eight negative results, four of the pools had pH values below 3.7 which might explain the absence of neutrophilic organisms. However, two other springs also below pH 3.7 (Rt71 and Wai9) gave positive enrichments. Positive enrichments might have been expected from the remaining four springs on the basis of their pH and temperature profile, but repeated attempts at enriching from springs Rt3 and Rt8 were unsuccessful. This does not indicate that such springs are sterile since both Rt3 and Rt8 possess a diverse flora of thermophilic eubacteria (Patel 1984; Patel et al. 1985b).

Seven pure cultures were obtained for further investigation, five rods (Ket10-S1, Rt13-S1, Tok12-S2, Wkv6-S1, and Tp44-S1) and two coccal (Rt59-S1 and Tok12-S1). Salient properties of the cultures are compared with reported values for *Thermoproteus* and *Desulfurococcus* isolates from Iceland (Table 2).

All the five rod shaped isolates resembled *Thermoproteus tenax* Kral in morphology (Zillig et al. 1981). They were 3–20 µm by 0.4 µm in dimension and formed terminal spherical protrusions, termed golf balls, in exponential growth phase. During approach of the stationary phase, constriction of the cytoplasm occurred and refractile bodies, usually more than one per cell, were also observed.

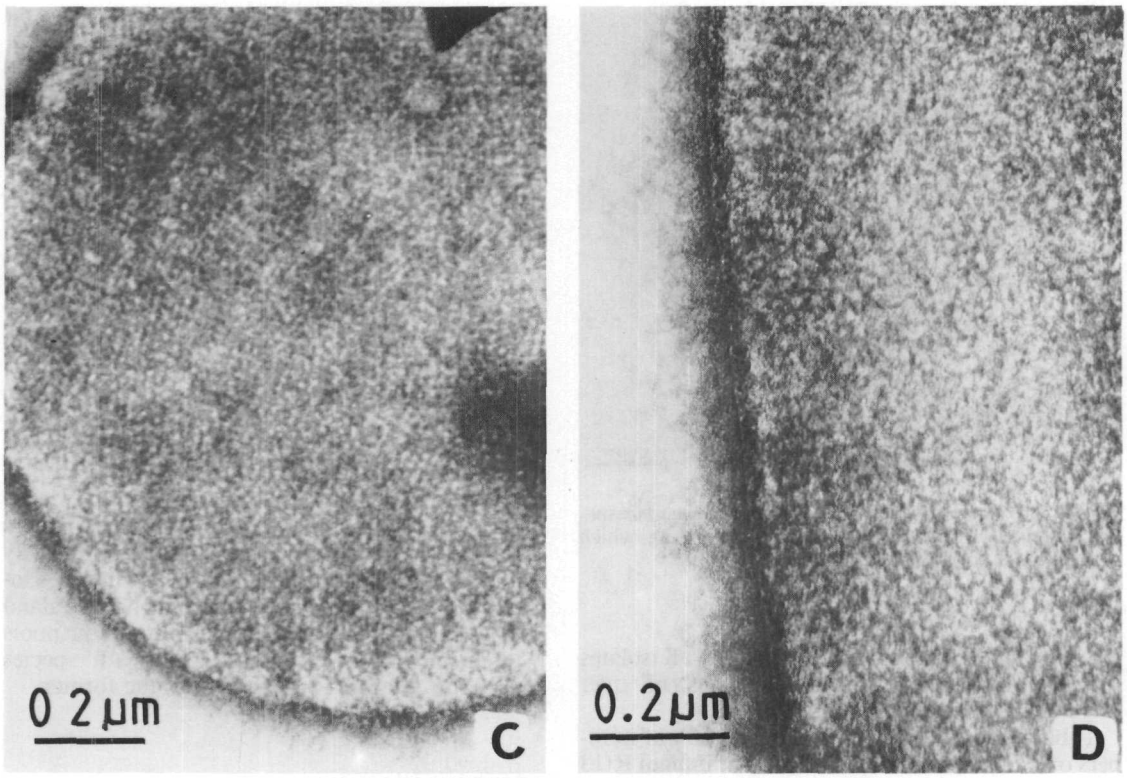


Fig. 1 An electron micrograph showing the association of a coccus and a rod-shaped archaebacterium observed in an enrichment culture obtained from pool Rt42 (A). The coccus possessed flagella (B) and the cell envelope possessed a distinct sub-unit structure (C) which appeared similar to that of the rod-shaped bacterium (D).

Electron microscopy of negatively stained preparations revealed that all the five isolates had cell walls made of distinct subunits (similar to that exemplified in Fig. 1D) which were masked by slime production in the late stationary phase of growth.

The temperature optima of the New Zealand isolates were between 88 and 90°C and similar to the temperature optima of other *Thermoproteus tenax* species (Fischer et al. 1983). However, the New Zealand isolates grew optimally at pH 6.0 to 6.9; this differs from *Thermoproteus tenax* (Zillig et al. 1981; Fischer et al. 1983) which grew optimally at a pH of 5.5 with no growth observed at pH 7.0. The New Zealand isolates also grew more slowly with generation times between 5.5 and 13.5 h. These features of the five isolates were similar to *Thermoproteus neutrophilus* (Fischer et al. 1983). However, *T. neutrophilus* is an obligate autotroph but all five New Zealand isolates were either facultative autotrophs or strict heterotrophs (Table 2). This is the first report on the isolation of strictly heterotrophic *Thermoproteus* species from geothermal environments. It would be interesting to investigate whether they are unique to New Zealand geothermal habitats.

No increase in cell numbers was obtained when all the five New Zealand isolates and *T. neutrophilus* were grown on acetate, ethanol, lactose, glucose, pyruvate, or sucrose as carbon sources. Additionally, *T. tenax* Kral in our hands was found not to utilise glucose and ethanol as carbon sources though Zillig et al. (1981) have reported that it does. With the exception of the obligate autotroph *T. neutrophilus,* all cultures showed good growth on Db medium with yeast extract as sole carbon source, i.e., no peptone, and an increase in cell numbers was evident at yeast extract concentrations of only 0.01%. When different carbon sources were added to Db medium the yeast extract must be reduced to below 0.01% to avoid misinterpretation of the carbon source actually used. Malate was the only substrate used as a sole carbon and energy source by the New Zealand isolates and *T. tenax* Kral but not by *T. neutrophilus.* Under opti-

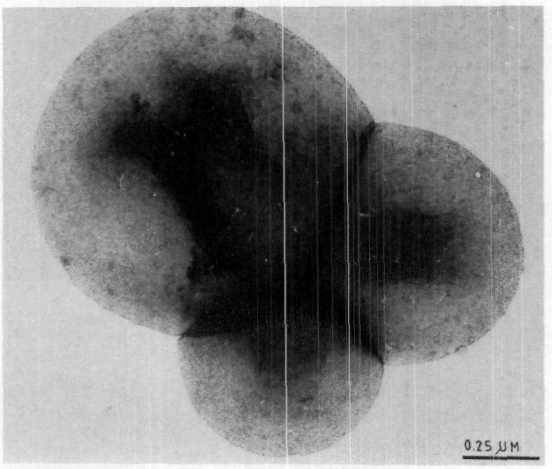


Fig. 2 An electron micrograph of a *Desulfurococcus* species obtained from a New Zealand hot spring which reproduces by budding.

mum growth conditions on Db medium all isolates produced maximum culture densities of over 1×10^8 cells ml^{-1} after 48 h incubation.

Table 3 presents data on the estimation of numbers of *Thermoproteus* species present in pool Rt13 (sediment or pool water) at incubation temperatures of 88 and 95°C in Db medium. Slightly higher values were obtained at 88°C for both water and sediment estimations than at 95°C. This was in accordance with the known optimum temperature for growth of both *T. tenax* (85°C) and for pure cultures (88–90°C) from New Zealand thermal pools (Table 2).

The greater number of cells found in pool sediments might indicate a lower Eh and/or higher substrate concentration in this environment. If the rods were adsorbed to sediment particles then the MPN approach might significantly underestimate the number of cells in the sediment, because the number of cells per particle could be high.

The New Zealand coccal strains closely resemble *Desulfurococcus* species. The New Zealand coccal isolates were Gram-negative, non-motile, and varied in diameter from 2.0–7.0 µm. Reproduction was by budding (Fig. 2). In late stationary phase an increasing number of cells appeared as membrane ghosts. Motility is a feature of *D. mobilis* but not *D. mucosus* and hence the New Zealand isolates appeared to be similar to *D. mucosus* rather than *D. mobilis*. However, both New Zealand isolates had slightly higher temperature optima than *D. mucosus* and isolate Rt59-S1 had a significantly different pH optimum (Table 2). Both New Zealand strains were unable to grow in media without either sulphur or cystine as electron acceptor. This is in contrast to observations by Zillig et al. (1982) on sulphur free growth of *D. mucosus*. Yeast extract did not support growth of either of the coccal strains, both of which had an obligate requirement for either peptone or casein.

All rod and coccal New Zealand isolates were deemed to be archaebacteria as a consequence of mode of reproduction (golf balls and budding respectively), their anaerobic respiration of sulphur, morphology, and ultrastructure in relation to type species and resistance to antibiotics particularly rifampicin and those inhibiting peptidoglycan synthesis (Jasperse-Herst 1984).

The geographic distribution of thermophilic archaebacteria is interesting. The *Thermoproteus* isolates obtained from pools in Iceland and USA have all been autotrophs or facultative autotrophs, the three New Zealand strains are the only ones reported incapable of autotrophic metabolism. *Desulfurococcus* has only been isolated previously from Icelandic hot springs, and could not be isolated from pools in Italy or Japan. The New Zealand pools would appear to be similar to Icelandic pools in their archaebacterial flora, but with species adapted to the more neutral pH of the former.

ACKNOWLEDGMENT

We gratefully acknowledge financial support from a BP EMRA award to Mrs Jasperse-Herst.

REFERENCES

APHA. Water Pollution Control Federation 1973: Standard methods for the examination of water and wastewater, 13th ed. Washington. pp. 672–676.

Brock, T. D. 1978: Life in boiling water. *In*: Thermophilic microorganisms and life at high temperatures. New York, Berlin, Heidelberg, Springer Verlag. pp. 302–336.

Buonocore, V.; Sgambati, O.; De Rosa, M.; Esposito, E.; Gambacorta, A. 1980: A constitutive β-galactosidase from the extreme thermoacidophile archaebacterium *Caldariella acidophila*: properties of the enzyme in the free state and in immobilized whole cells. *Journal of applied biochemistry 2*: 390–397.

Fischer, F.; Zillig, W.; Stetter, K. O.; Schreiber, G. S. 1983: Chemolithotrophic metabolism of anaerobic extremely thermophilic archaebacteria. *Nature 301*: 511–513.

Fox, G. E.; Stackebrandt, E.; Hespell, R. B.; Gibson, J.; Maniloff, D.; Dyer, T. A.; Wolfe, R. S.; Balch, W. E.; Tanner, R. S.; Magrum, L. J.; Zablen, L. B.; Blakemore, R.; Gupta, R.; Bonen, L.; Lewis, B. J.; Stahl, D. A.; Luehrsen, K. R.; Chen, K. N.; Woese, C. R. 1980: The phylogeny of prokaryotes. *Science 209*: 457–463.

Jasperse-Herst, P. M. 1984: Isolation and characterization of extremely thermophilic archaebacteria from New Zealand hot springs. Unpublished M Phil thesis, University of Waikato, Hamilton, New Zealand.

Patel, B. K. C. 1984: Extremely thermophilic bacteria in New Zealand hot springs. Unpublished PhD thesis, University of Waikato, Hamilton, New Zealand.

Patel, B. K. C.; Morgan, H. W.; Daniel, R. M. 1985a: *Fervidobacterium nodosum* gen. nov. and sp. nov., a new chemo-organotrophic, caldoactive, anaerobic bacterium. *Archives of microbiology 141*: 63–69.

———— 1985b: Thermophilic anaerobic spirochetes in New Zealand hot springs. *FEMS microbiology letters 26*: 101–106.

———— 1985c: A simple and efficient method for preparing and dispensing anaerobic media. *Biotechnology letters 7*: 277–278.

———— 1986: Unusual microorganisms observed in New Zealand hot springs. *Microbial ecology* (in press).

Stetter, K. O.; Thomm, M.; Winter, J.; Wildgruber, G.; Huber, H.; Zillig, W.; Janekovic, D.; König, H.; Palm, P.; Wunderl, S. 1981: *Methanothermus fervidus* sp. nov., a novel extremely thermophilic methanogen isolated from an Icelandic hot spring. *Zentralblatt für bakteriologie und hygiene. I. Abteilung originale, C2*: 166–178.

Stetter, K. O.; König, H.; Stackebrandt, E. 1983: *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulphur reducing archaebacteria growing optimally at 105°C. *Systematic and applied microbiology 4*: 535–551.

Zillig, W.; Stetter, K. O.; Schafer, W.; Janekovic, D.; Wunderl, S.; Holz, I.; Palm, P. 1981: Thermoproteales: A novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from Icelandic solfataras. *Zentralblatt für bakteriologie und hygiene I, Abteilung originale C2*: 205–227.

Zillig, W.; Stetter, K. O.; Prangishvilli, D.; Schafer, W.; Wunderl, S.; Janekovic, D.; Holz, I.; Palm, P. 1982: Desulfurococcaceae, the second family of the extremely thermophilic anaerobic sulphur-respiring Thermoproteales. *Zentralblatt für bakteriologie und hygiene I. Abteilung originale C3*: 304–317.

Zillig, W.; Gierl, A.; Schreiber, G.; Wunderl, S.; Janekovic, D.; Stetter, K. O.; Klenk, H. P. 1983a: The archaebacterium *Thermofilum pendens* represents a novel genus of the thermophilic, anaerobic sulphur respiring Thermoproteales. *Systematic and applied microbiology 4*: 79–83.

Zillig, W.; Holz, I.; Janekovic, D.; Schaefer, W.; Reiter, W. D. 1983b: The archaebacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of archaebacteria. *Systematic and applied microbiology 4*: 88–94.