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Isolation of an extremely thermophilic chemoorganotrophic anaerobe similar to *Dictyoglomus thermophilum* from New Zealand hot springs

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Abstract. A strain similar to *Dictyoglomus thermophilum*, isolated from a New Zealand hot spring, is described. This strictly anaerobic, Gram-negative, non-motile and non-sporulating bacterium usually exists as long thin filaments of 5 to 25 μm by 0.35 to 0.45 μm. Rotund bodies are commonly observed. Thin sections of the cells revealed a two-layered cell wall. The optimum temperature and pH for growth was 70° C and 7.0 and 7.5 respectively. No growth was observed at 40° C and 85° C or at pH 4.5 to pH 9.0. The organism fermented glucose, maltose, mannose, xylose, lactose, cellobiose, galactose and sucrose and produced acetate as the major end-product with significant amounts of lactate, H_2 and CO_2 and only traces of ethanol. The doubling time on glucose was 10 h. The DNA base composition was 29.5% guanine plus cytosine as determined by the thermal denaturation method. Growth was inhibited by penicillin, tetracycline and chloramphenicol indicating that the organism was a eubacterium. These features are in common with the newly described species *Dictyoglomus thermophilum* to which the New Zealand isolate belongs.

Key words: *Dictyoglomus* – *Fervidothrix* – Description – Rotund bodies – Extremely thermophilic – Obligate anaerobe – Fermentation products – Physiology – Distribution

An increasing number of extremely thermophilic and strictly anaerobic glucose-utilising bacteria capable of rapid growth with short doubling times and high densities under optimal conditions, have been isolated from geothermal environments (Zeikus et al. 1979; Morgan et al. 1985; Wiegel and Ljungdahl 1981; Ben-Bassat and Zeikus 1981; Patel et al. 1985a). During our investigations on the ecology of extremely thermophilic anaerobic bacteria from New Zealand hot springs, we isolated a novel extremely thermophilic, filamentous and strictly anaerobic bacterium which grew extremely slowly with very low cell yields. It was tentatively described as "*Fervidothrix*" (Patel et al. 1986a) but subsequently a similar organism was isolated from a Japanese hot spring and named *Dictyoglomus thermophilum* (Saiki et al. 1985). The communication presented here, is to the best of our knowledge, the first report on the isolation and characterization of *Dictyoglomus* species from New Zealand hot springs, and extends the range of fermentation end-products previously reported.

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Materials and methods

All the media used for enrichments have been described in Table 1. Unless indicated, methods of sampling geothermal areas, anaerobic media preparation, enrichment, isolation, cellular and metabolic studies have been described elsewhere (Patel et al. 1985a, b, c, 1986a, b; Morgan et al. 1985). Fermention end-products were analysed as previously described (Patel et al. 1985a) except that volatile fatty acids and alcohols were determined on chromosorb 101, 80–100 mesh (Sigma Chemicals Co., St. Louis, MO, USA) at a anaerobic glucose-utilising bacteria capable of rapid growth flow rate of 24 ml/min. Lactate was determined enzymatically as described by Gutmann and Wahlefeld (1974). Continuous culture studies were performed using LH fermentation equipment (L.H. Engineering Co. Ltd., Bucks., UK). The type culture of *Dictyoglomus thermophilum* H-6-12 (= ATCC 35947) was kindly provided by Dr. T. Beppu, Department of Agricultural Chemistry, The University of Tokyo, Tokyo, Japan.

Results and discussion

Ninety five enrichments were obtained from the 370 geothermal spring samples which had been inoculated in TYEG medium. Although several enrichments contained rods with spheroids (Patel et al. 1985a), three other enrichments appeared unique in that extensive filaments were associated with rotund bodies. Cultures from these three enrichments were purified and designated Rt46-B1, Rt8N2 and Tos-80-la-d (Table 1).

Although the culture designated RtN82 was enriched on a medium containing acetate and sodium triphosphate omission of these components had no effect on growth, and better growth was recorded on TYEG medium. Similarly, Tos-80-la-d although enriched on cellulose, did not grow on it and nor did it posses cellulase activity.

In general, all these three isolates were strict anaerobes and no growth was observed in an anaerobic medium which was partially oxidised as determined by the pale pink color of resazurin.

The isolates usually occurred as filaments of 5 to 25 μm in length by 0.35 to 0.45 μm in diameter. Mini cells though present, were rarely observed. Spores were not observed at any stage during these studies. The filaments sometimes

Table 1. Thermal features of geothermal springs yielding filamentous organisms with rotund bodies

Pool no. and description of pool	Temperature (°C)	pH	Strain designation
Pool Rt8; Outflow from a vent in Government Gardens; algal and bacterial mats present[a]	64	9.0	Rt8N2
Pool Rt46; Un-named bubbling and shallow spring with sandy sediment in Kuirau Park[b]	90	2.8	Rt46-B1
Pool Tos 80; Outflow of un-named bubbling pool in Federigo solfatara, Tuscany, Italy[c]	55–85	7.5	Tos-80-la-d

Springs from which such organisms could not be isolated included all New Zealand geothermal areas other than Rotorua (Waimangu, Taupo, Tokaanu, Orakei Korako, Waiotapu, Tikiteri, Wairakei, White Island and Hot Water Beach), Fiji Islands, Iceland, Taiwan and USA

Enrichments were initiated in:

[a] Medium N contained g per l of distilled water NH_4Cl, 0.25; $MgCl_2 \cdot 6\,H_2O$, 0.4; KH_2PO_4, 0.2; Na_2SO_4, 3.0; NaCl, 1.0; KCl, 0.5; trace mineral solution, 9 ml (Zeikus and Wolfe 1972); 0.2% resazurin, 1 ml; 0.5 ml of vitamin solution (Zeikus and Wolfe 1972); $Na_2S \cdot 9\,H_2O$, 1; yeast extract, 0.2; trypticase peptone, 0.2; sodium acetate, 3.0 and sodium triphosphate, 5.0

[b] Trypticase-yeast extract-glucose (TYEG) media (Zeikus et al. 1979)

[c] Medium T contained g per l of distilled water. KH_2PO_4, 0.68; NH_4Cl, 0.3; NaCl, 0.3; $CaCl_2 \cdot 2\,H_2O$, 0.11; $MgCl_2 \cdot 6\,H_2O$, 0.1; $NaHCO_3$ 1.0 g; trace element solution, 1 ml and vitamin solution, 1 ml. The trace element solution contained per l of distilled water $FeCl_2 \cdot 4\,H_2O$ 2.0 g; 0.05 g each of H_3BO_3, $ZnCl_2$, $MnCl_2$, $(NH_4)_6Mo_7O_{26} \cdot 4\,H_2O$, $AlCl_3$, $CoCl_2 \cdot 6\,H_2O$, $NiCl_2$; EDTA, 0.5 g; $CuCl_2$, 0.03 g and HCl, 1 ml. The vitamin solution contained per l 2 mg each of Biotin and Folic acid; 5 mg each of pyrodoxine-HCl, riboflavin, thiamine-HCl, nicotinamide, cyanocobalamin, p-aminobenzoic acid, thioctic acid and pantothenic acid. The first enrichment contained avicel cellulose (0.5%) as a carbon source and subsequent subcultures contained glucose (0.2%)

existed with terminal loops or as coiled spring-like cells. The filaments aggregated together to form ball-like structures known as rotund bodies. The size of the rotund bodies varied between 3 to 20 μm in diameter. Rotund bodies were more prevalent during unfavourable conditions e.g. late logarithmic or stationary phase, at low pH (5.5), high pH (8.0) and at high temperatures (75 to 78°C). Rotund bodies were observed to degenerate in which the cells within collapsed and formed extremely small spherical structures, which were observed to undergo Brownian motion.

The filaments possessed a two layered cell-wall. The outer layer formed the retaining membrane of the rotund body structure in which groups of cells were encompassed. Whether rotund bodies form due to cell division of the cell within or due to fusion of the outer cell walls of a number of cells or by a combination of both is not known.

As all the three strains resembled each other morphologically, additional studies were conducted on isolate Rt46-B1 only. Isolate Rt46-B1 was extremely sensitive (10 μg/ml) to penicillin, tetracycline, chloramphenicol and neomycin, less sensitive (100 μg/ml) to D-cycloserine and tolerant (500 μg/ml) to sodium azide. This indicates that isolate Rt46-B1 is a eubacterium and not an archaebacterium (Zillig et al. 1983).

The New Zealand isolate Rt46-B1 has a G+C content of 29.5% which is similar to the G+C content of a number of other extremely thermophilic eubacterial anaerobes (Patel et al. 1985; Wiegel and Ljungdahl 1981; Zeikus et al. 1979; Saiki et al. 1985). However, it most closely resembles *F. nodosum* (Patel et al. 1985) and *D. thermophilum* (Saiki et al. 1985) mainly in its ability to form rotund bodies. Rotund bodies are also produced by *Thermus* species but *Thermus* is an aerobe and has a much higher G+C content of approximately 61 to 70% mole G+C (Brock and Freeze 1969). *F. nodosum* however has a distinctly different cell morphology in that it produces terminal spheroids and also produces different end-products and end-product ratios. Hence we believe isolate Rt46-B1 is a strain of *Dictyoglomus thermophilus* species.

The pH optima and range for growth is also similar to *Dictyoglomus thermophilus* species. The optimum pH for growth of isolate Rt46-B1 was about 7.5. Growth also occurred at pH 6.0 and 8.5 but not at pH 4.5. The optimum temperature for growth was 70°C in batch tube culture [in the medium described by Saiki et al. (1985) or in TYEG medium] or in continous culture (in TYEG medium) whereas that of *D. thermophilum* is 78°C. Despite the differences in optimum growth temperature this is not reflected in maximum temperature for growth, both cultures showing growth at 82°C but not at 85°C.

The maximum specific growth rate of $D = 0.14\ h^{-1}$ was obtained at 70°C under continous culture. The cellular yield (g/l) of isolate Rt46-B1 grown under continous culture ($D = 0.14\ h^{-1}$) at 65°C was 0.29 and at 70°C was 0.32. Complete washout of isolate Rt46-B1 occurred at 79°C under this condition.

Isolate Rt46-B1 and *D. thermophilum* are capable of growing albeit poorly, on trypticase peptone and yeast extract (TYE) medium. However, the growth yield increases on addition of a fermentable carbohydrate such as glucose (Table 2). Yeast extract or peptone could not be replaced with a vitamin mixture and/or with a vitamin free casamino acid mixture. The vitamins required by *C. thermocellum* (Johnson et al. 1981) and *C. thermoaceticum* (Lundie and Drake 1984) are available in this defined medium. Increasing the yeast extract concentration from 0.3% to 0.5% or above during fermentation on glucose, inhibited growth of isolate Rt46-B1.

The end-products of glucose fermentation include ethanol, acetate, lactate, H_2 and CO_2. A true fermentation balance of glucose utilization by isolate Rt46-B1 and *D thermophilum* could not be achieved because of the interference caused by similar products being formed from the utilization of yeast extract. Typical end-product kinetics of isolate Rt46-B1 during fermentation on glucose are shown in Fig. 1. All end-products increased in response to growth and glucose consumption.

A number of other carbohydrates are also fermented by isolate Rt46-B1 and *D. thermophilum* and the growth rates are shown in Table 2. The generation times varied from 7 h to 17 h depending on the type of carbohydrate fermented. The final pH after fermentation also varied (from between 5.3 to 7.0) and again was dependent on the type of carbohydrate fermented. Acetate was always the major end-product formed during fermentation (Table 2). In general, the Japanese isolate produced marginally more acetate on

Table 2. Comparison of carbohydrate utilization and end-product formation by NZ isolate Rt46-B1 and *D. thermophilum*

Carbohydrate tested[a]	Isolate Rt46-B1				*D. thermophilum*			
	OD (660 nm)	pH	Acetate produced (mM)	Generation Time (h)	OD (660 nm)	pH	Acetate produced (mM)	Generation Time (h)
Lactose	0.14	7.0	6.5	17.0	0.14	6.9	6.8	17.0
Cellobiose	0.24	6.8	9.0	15.0	0.21	6.6	10.9	12.5
Sucrose	0.29	6.3	13.9	10.0	0.25	5.8	20.4	10.0
Maltose	0.40	5.9	21.5	10.0	0.25	6.2	15.9	7.5
Xylan[b]	ND	5.3	19.8	ND	ND	5.4	18.9	ND
Glucose	0.28	6.5	12.9	10.0	0.31	5.7	16.9	10.0
Mannose	0.30	6.5	14.4	15.0	0.26	5.8	18.8	11.5
Galactose	0.34	6.4	13.5	12.5	0.27	5.9	17.3	10.0
Potato starch[c]	0.28	6.3	12.0	10.0	0.41	6.2	17.5	7.0
Arabinose	0.09	6.5	8.4	ND	0.11	6.2	13.7	10.0
TYE control	0.08	7.1	5.1	ND	0.10	7.1	6.2	ND

[a] To 13 ml of TYE medium, carbohydrates from sterile stock solutions were added to a final concentration of 0.5%. The tubes were inoculated with isolate Rt46-B1 or *D. thermophilum* (10% inoculum), incubated at 70°C and 75°C respectively and growth followed by measuring optical densities at various time intervals. pH and acetate were measured at the end of the growth phase. None of the isolates grew on rhamnose and cellulose and acetate production equalled that of the TYE control tubes. No autotrophic ($CO_2 + H_2$) growth was observed

[b] An active xylanase was detected

[c] An active amylase was detected

ND = not determined

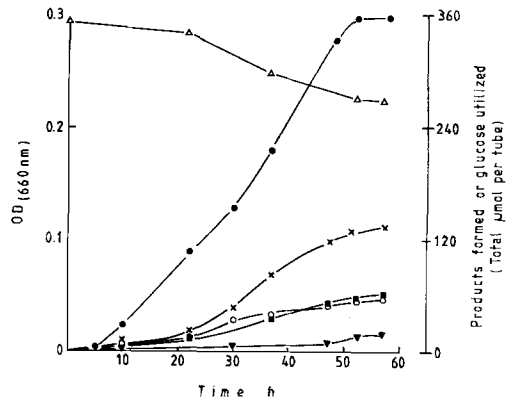

Fig. 1. Relationship of end product formation to growth of isolate Rt46-B1. Anaerobic Bellco tubes containing 13 ml of TYEG medium, pH 7.2 were inoculated from a culture obtained from continuous culture and the tubes incubated at 70° C. The results are given as micromol per tube at indicated times. Symbols: ▼——▼ lactate; ○——○ H_2; ■——■ CO_2; ×——× acetate; ●——● growth; △——△ glucose utilized

most substrates than the New Zealand isolate which was reflected in lower culture pH and faster generation times. Although Saiki et al. (1985) reported the production of only acetate, lactate and CO_2 on glucose fermentation, we find that both cultures consistently produced in addition small amounts of ethanol and significant amounts of hydrogen. We were also unable to reproduce the much lower generation times recorded by Saiki et al. (1985), but this may reflect the different methods of measuring growth rates. Given these similarities we propose prior designation of our isolate as a new genus "*Fervidothrix*" to be unwarranted (Patel et al. 1986a), and that it be regarded as a strain of *Dictyoglomus thermophilum*.

The distribution of *Dictyoglomus* sp. appears to be restrictive and warrants further study. Saiki et al. (1985) obtained a positive enrichment in only one of the six springs sampled. In our survey only three positive enrichments from 370 springs were obtained, the isolation from spring Rt46 was unexpected, given the low pH and high temperature of the water. For pools Rt8 and Tos-80 the parameters are closer to those recorded for the Japanese spring. From the present survey we must assume the habitat of *Dictyoglomus* to be very restrictive in that they were isolated from only a single thermal region of New Zealand. Many of the springs from New Zealand and other thermal areas (Table 1) covered the same pH/temperature profile as Rt8, Tos 80 and Tsuetate hot springs and many supported the growth of a mixed anaerobic flora. It is unlikely that that the presence of *Dictyoglomus* species could have been overlooked since long incubation schedules together with microscopic examination of positive enrichment cultures would have picked up the peculiar morphological features of these organisms with relative ease. However, the possibility that *Dictyoglomus* species may be inhibited by other fast growing thermophiles cannot be ruled out. We must assume that factors other than temperature and pH limit the distribution in hot springs.

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