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Isolation and characterization of a strictly xylan-degrading *Dictyoglomus* from a man-made, thermophilic anaerobic environment

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Abstract. A thermophilic, strictly anaerobic eubacterium which utilized an unusually limited range of substrates was isolated from a sludge and pulp sample from a paper-pulp cooling tank at a paper-board factory in Finland. The organism grew only with beech wood or oat spelt xylan; no growth occurred with soluble sugars, other polysaccharides, peptone, or yeast extract. The organism was rod-shaped, long (up to 20 μm), thin (0.3 μm), gram-negative, and in late-exponential and stationary phase cultures formed "ball of yarn" like structures; endospores were not observed and the organism was not motile. The organism grew fastest ($\mu = 0.08-0.09\ h^{-1}$) at 65 to 75° C and pH 6.5 to 8.4, with a maximum growth temperature between 75 and 80° C and an upper pH limit near 9. During growth on beech xylan the isolate produced only acetate, H_2, and CO_2 as fermentation products. The guanine + cytosine (G + C) content of the isolates cellular DNA was 34%. The unusual morphology of the isolate is characteristic of the genus *Dictyoglomus*, and the limited substrate range, higher G + C ratio, and different fermentation products indicated that the isolate was a new species in that genus.

Key words: *Dictyoglomus* – Anaerobe – Thermophilic – Xylan

In the last twenty years there has been increased interest in and study of microorganisms from extreme environments, particularly bacteria from thermophilic environments (Bergquist et al. 1985; Brock 1985). They are being examined with attention to not only their special biology, which enables them to survive and prosper in an extremely harsh environment, but also the potential for employment of their thermostable products, such as hydrolytic enzymes, in technological processes (Bergquist et al. 1985; Zeikus 1979).

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As xylan is a very common material, it can occur naturally in thermophilic environments. Leaves, grass, and wood all contain xylan and can be blown or washed into hot springs and several species of xylan-degrading, thermophilic anaerobes from hot-springs have been described. Xylan-degrading species are found in the genera *Clostridium, Thermoanaerobium, Thermoanaerobacter*, and *Thermobacteroides* (Wiegel et al. 1985).

A relatively new group of thermophilic anaerobes, and microorganisms with a distinctive morphology, are members of the genus *Dictyoglomus* (Sakai et al. 1985). *Dictyoglomus* species form an unusual spherical structure previously reported only in thermophilic aerobes (Brock and Freeze 1969). There are only three reported isolates of *Dictyoglomus* (Patel et al. 1987; Sakai et al. 1985; Svetlichnii and Svetlichnaya 1988) and only one validly published species, *D. Thermophilum* (Sakai et al. 1985). All extant isolates are from natural thermophilic environments and use many different fermentable carbohydrates for growth. We describe here the isolation of an unusual, strictly xylan-degrading strain of *Dictyoglomus* from a xylan-rich man-made thermophilic environment.

Materials and methods

The medium used was as previously described (Angelidaki et al. 1990), with the following differences; beech wood xylan (Novo Nordisk, Bagsværd, Denmark) was added as substrate, 4 g/l; cysteine was not added; sulfide was increased to 0.5 g/l; and the vitamin solution, 10 ml/l, was that of medium no. 141 of the German Collection of Microorganisms and Cell Cultures (DSM). Different pH values in the medium were obtained by varying the concentrations of HCO_3^- in the medium and the CO_2 content of the headspace gas; the buffer was necessarily weak at higher pH values but all growth rate measurements were obtained early in the growth curve and the pH was measured to verify that it had not changed significantly. Unless otherwise stated, cultures were grown in the dark without shaking at 68° C. The turbid, xylan-containing medium interfered with cell measurement by turbidometric methods and protein measurement, so specific growth rates, μ (h^{-1}), for the identification of the optimum temperature and pH for growth, were determined from the specific acetate production rate. The correlation of acetate production and cell number was determined by

counting cells in a Petroff-Hauser counting chamber after treatment with 0.025% sodium dodecylsulfate to dissolve the spherical structures and separate the cells from each other (Svetlichnii and Svetlichnaya 1988). Fermentation products were identified by gas chromatography with thermal conductivity detection for gases and flame ionization detection for alcohols and short chain fatty acids, except formate. HPLC with refractive index detection was used for measurement of lactate and formate. Residual xylan was measured gravimetrically using the method of Weimer and Zeikus (1977) for cellulose solubilization. Controls were performed to insure that xylan was not solubilized during formic acid treatment and results were compared to uninoculated controls incubated under the same conditions. Transmission electron micrographs of thin sections of late exponential phase cultures fixed with gluteraldyhyde in cacodylate buffer and stained with osmium tetraoxide (Huser et al. 1982) were taken with a Jeol 100 SX. The G + C (guanine + cytosine) ratio of the cellular DNA of the isolate was measured by HPLC (Meshbah et al. 1989) at the DSM. All experiments were repeated at least twice; average values from all experiments are reported and error bars on figures represent the range of at least three values from a typical experiment.

Results

Samples

A sample of pulp-mass and water from a pulp-mass cooling tank was collected at the Metsä-Serla neutral sulphite semi-chemical process, paperboard-carton pulp-factory in Kirkniemi, Finland. The temperature in the tank was 70 – 80° C, the pH was 5.5 – 6.5, and the pulp-mass residence time in the tank was about 3.5 h. Volatile fatty acids were being produced in the tank, indicating anaerobic microbial activity, and microscopic examination of the sample showed many different forms of bacteria. The most unusual morphological form was a long, very thin, rod-shaped organism.

Enrichment cultures

Enrichment cultures with medium at pH 6, 7, 8.3 and 9, and 4 g/l beech-tree or oat-spelt xylan as substrate were inoculated (100 ml/l) with aliquots of the pulp-mass sample and incubated at 68° C without shaking. Growth occurred within one week in the cultures with pH 6.0, 7.0, and 8.3 as indicated by solubilization of the xylan, a visible and large increase in the number of microbes in the culture, production of gas which produced overpressure in the bottles, the detection of methane in some cultures, and the accumulation of acetate, up to approx. 25 mM, or ethanol. All growth indicators were compared to inoculated controls without substrate. The enrichment cultures were transferred several times in the same media. The most common cell type in the culture at pH 8.3 with beech xylan was a long, thin rod (up to 15 µm × 0.3 µm) which formed spherical structures. During growth the pH in the medium decreased from 8.3 to approx. 7.9.

Isolation

We isolated several xylan-degrading organisms from enrichment cultures with pH 7.0 or 8.3 and beech or oat xylan as substrate. Organisms were isolated in anaerobic roll-tubes solidified with Gelrite and with beech-wood or oat xylan as substrate and the pH as in the respective enrichment culture. Large clearing zones (up to 1 cm diameter after one week incubation at 68° C) in which the xylan had been solubilized were present around colonies of xylan degrading organisms, indicating the release of extracellular enzyme. Colonies were picked with sterile, anaerobic Pasteur pipettes and transferred to the same medium and incubated; the process was repeated until only one colony type was observed. Two very similar *Dictyoglomus*-like strains were isolated from the beech-wood xylan enrichments at pH 8.3, strain B1 is described here.

Colony and cell morphology

Surface colonies of strain B1 in anaerobic roll tubes were tan and circular with an entire edge; subsurface colonies were lens-shaped. The colonies had an iridescent, grainy interior and microscopic examination of picked colonies showed that the long, thin cells of the culture lay side by side in extensive, parallel ranks.

Liquid cultures of strain B1 in early exponential growth phase contained rod-shaped cells with rounded ends, 5 to 20 µm in length, and approx. 0.3 µm wide. Cells occurred singly and in pairs and bundles laying side by side. Cells in exponential phase did not stain gram-positive but did not bind safranin well, and thus were nearly colorless after gram Stain preparation. Endospores were not observed and the organism was not motile. Cultures did not spontaneously form spheroplasts and spheroplasts were not induced by lysozyme, however, cells were sensitive to lysis by lysozyme at concentrations over 0.25 g/l as judged by microscopic observation. In late exponential phase cultures, the cell bundles were often swollen and intermediate forms up to spherical "ball of yarn" structures, with cells laying on the surface of the sphere were observed (Fig. 1). The spherical structures were from 5 to 25 µm in diameter and examination at high magnification showed that the ball structure had a membrane or wall. In older cultures, cells could be seen peeling away from the ball structures and naked spheres of all sizes were present. In exponential phase cultures the ball structures were empty. In stationary phase cultures, a small fraction of the larger spheres, particularly those from which cells had peeled off, contained small amounts of heterogenous material which looked like cell detritus. Occasionally, inside of a mother sphere from which cells had peeled off, one or several smaller daughter spheres were observed. The spherical structures were sensitive to lysis by sodium dodecyl sulfate at 0.25 g/l or more, but the cells were stable; cells were whole, did not appear lysed, were well dispersed, and no bundles or spheres were visible.

Transmission electron micrographs

Transmission electron micrographs of late exponential phase cultures showed a single layered homogeneous cell

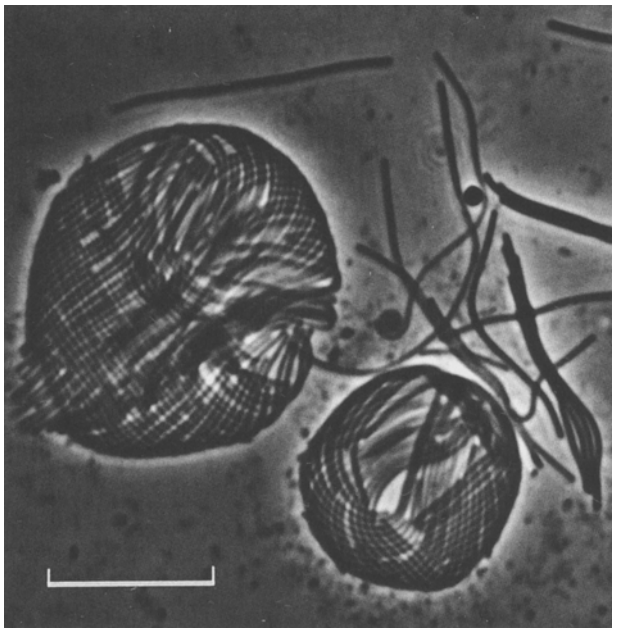

Fig. 1. Phase contrast photomicrograph of late exponential phase cells of strain B1. Reference *bar* is 10 μm

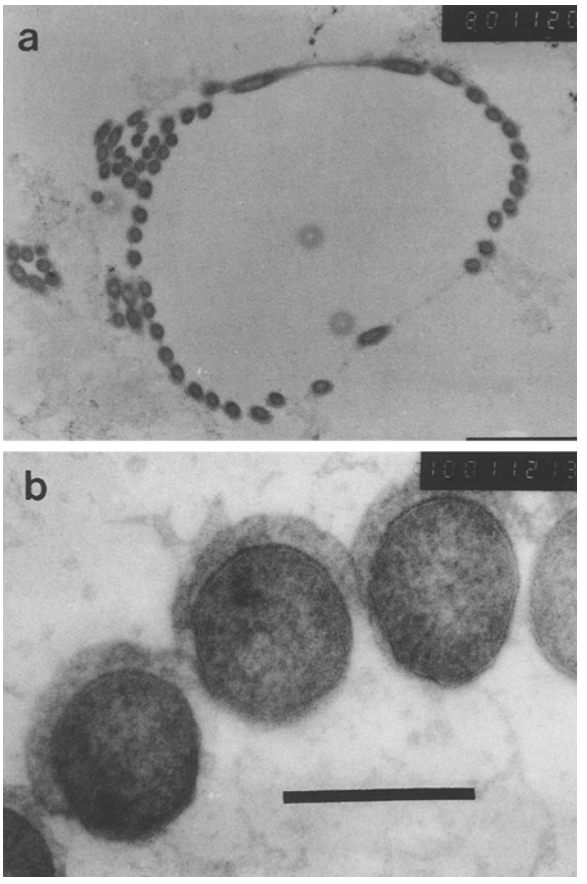

Fig. 2a, b. Transmission electron photomicrographs of late exponential phase cells of strain B1 in a spherical structure. Reference *bar* in **a** is 5 μm and in **b** is 0.25 μm

wall that was visible outside the bilipid layer of the cell membrane (Fig. 2). No special interior features within the cells were visible. Thin sections through ball structures or cells in bundles showed that the spheres were empty and that a thick cell-coat was present on the outer surface of the "ball of yarn" structures and cell bundles. The cell-coat was a diffuse material, not as dense as the cell wall, and was approximately 40 nm in diameter.

Substrates and nutritional requirements

Of all substrates tested, strain B1 would only use beech or oat spelt xylan at 4 or 1 g/l. There was no growth or metabolism detected, (as measured by culture optical density, production of fermentation products, and solubilization of the substrate) with xylose, xylobiose, arabinose, glucose, fructose, sucrose, galactose, mannose, maltose, rhamnose, lactose, soluble starch, cellobiose, microcrystalline cellulose, casein peptone, yeast extract,

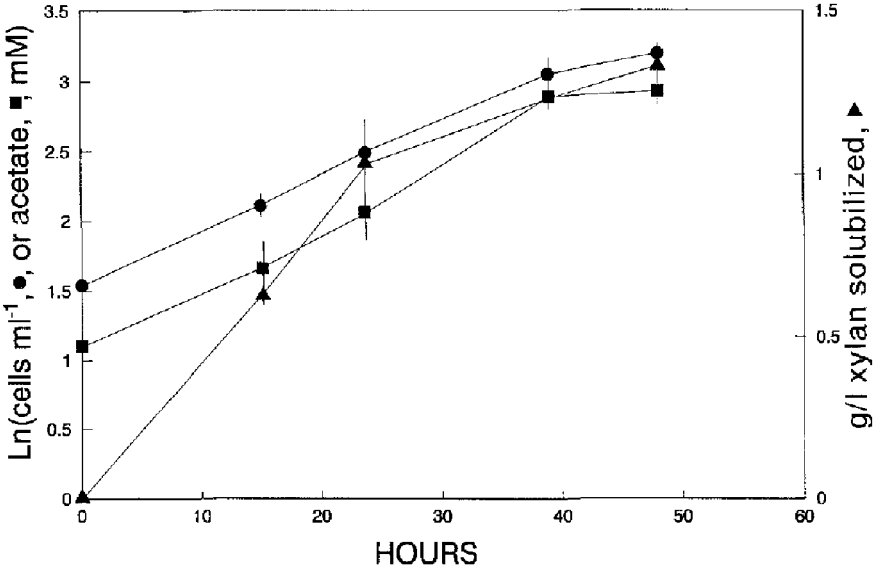

Fig. 3. Correlation of cell growth, acetate production and xylan degradation in beech xylan cultures of strain B1. Cell numbers are 10^7 ml^{-1}

Table 1. Characteristics of extant *Dictyoglomus* strains

Isolates	Substrates used	Fermentation products[a] (substrate)	G + C%	Source	Reference
B1	Xylans only	Ac(h)[a], H_2 (beech xylan)	34	Pulp mill cooling tank	Our isolate
Dictyoglomus thermophilum	Numerous carbohydrates[b]	Ac(l)[a], Lac(h[a]), H_2 (starch)	29	Hot-spring	Saiki et al. 1985
RT46-B1	Numerous carbohydrates[b]	Ac(h)[a], EtOH, Lac(l)[a], H_2 (glucose)	29.5	Hot-spring	Patel et al. 1987
D. turgidis Z-1310	Numerous carbohydrates[c] lignin, humic acids glycogen, peptone, CMC[a]	Ac(h)[a], EtOH, Lac(l)[a], H_2 (starch)	32.5	Hot-spring	Svetlichnii and Svetlichnaya 1988

[a] Ac = acetate; EtOH = ethanol; Lac = lactate; l = low concentration, less than approx. 5 mM; h = high concentration, more than approx. 5 mM; CMC = carboxymethylcellulose
[b] Xylan degradation reported (Patel et al. 1987)
[c] Does not use xylose, xylan degradation not tested

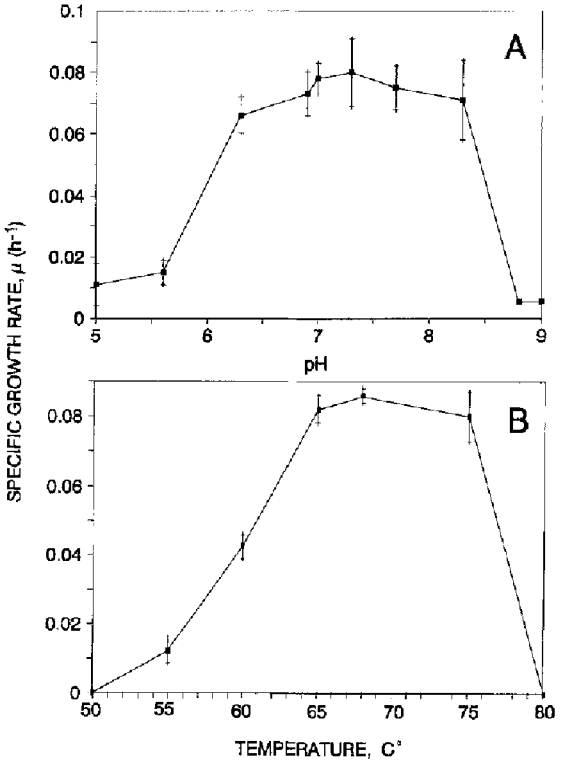

Fig. 4. The effect of pH (**A**) and temperature (**B**) on the specific growth rate of strain B1

or a syrup of acid-hydrolyzed xylan (Novo Nordisk, Bagsværd, Denmark), all at 4 or 1 g/l dry weight.

Growth was faster in the presence of both yeast extract (0.75 g/l) and vitamin solution (10 ml/l), however, neither were required for growth.

Fermentation products

The major fermentation products from beech xylan, at 4 g/l, were acetic acid, up to 55 mM, hydrogen gas, 1–2 mM, and carbon dioxide; lactate, formate, or ethanol were not detected (detection limit, 0.25 mM). There was a good correlation between the production of acetate, cell growth and degradation of xylan (Fig. 3).

Optimum pH and temperature for growth

Fastest growth for strain B1 at 68°C occurred from pH near 6 to over 8, with slow growth at pH 5.0 and little or no growth at pH 9 (Fig. 4). The effect of temperature on the growth rate of strain B1 is shown in Fig. 5. At pH 7.0, growth occurred from below 55°C to over 75°C, but there was no growth at 50 or 80°C. Fastest growth occurred from approx. 65°C to 75°C.

Antibiotic sensitivity

Strain B1 was sensitive to chloramphenicol, kanamycin, penicillin, streptomycin, tetracycline, and vancomycin, but not ampicillin, all at 100 mg/l. At 10 mg/l, strain B1 was unaffected by ampicillin, chloramphenicol, and tetracycline but was still sensitive to the other antibiotics previously tested at 100 mg/l.

Guanine plus cytosine ratio

The mol percent G + C ratio of the cellular DNA of strain B1 was 34.2 ± 0.3 as determined by HPLC.

Strain B1 has been deposited into the German Collection of Microorganisms and Cell Cultures (DSM, Braunschweig, FRG) as a patent strain.

Discussion

The antibiotic sensitivity pattern of strain B1 indicates that it is a eubacterium; it was not resistant to many

of the cell-wall-active antibiotics as are members of the *Archaeobacteria*.

The only other group of thermophilic anaerobes that shares the unusual morphological features of strain B1 are the members of the genus *Dictyoglomus*. The extant strains of *Dictyoglomus* are all strictly anaerobic, thermophilic, and carbohydrate fermenting like strain B1. Table 1 presents the major characteristics of strain B1 and all other *Dictyoglomus* isolates. Unlike any reported *Dictyoglomus* strain, or any reported xylan degrading thermophilic anaerobe, strain B1 used only xylans as substrate. This is a characteristic unique to strain B1. In addition, strain B1 made only acetate, H_2, and CO_2 as fermentation products and did not produce significant amounts of lactate, ethanol or formate. The DNA base composition of strain B1, 34%, is significantly higher than that of *D. thermophilum*, 29%, which is the type species and the only validly named species in the genus.

Preparation of wood chips for the production of paperboard pulp involves the solubilization of hemicellulose and lignins, at high pH and temperature, to separate them from the cellulose fibers. This process proceeds the cooling of the pulp-mass in the tank from which we received the pulp-mass sample and from which strain B1 was isolated: there is a continually replenished supply of free hemicelluloses, mostly xylans, in the cooling tank (personal communication, Armi Temmes Metsä-Serla Paper, Kirkniemi, Finland). To avoid competition with other organisms for the products of soluble xylosidases, strain B1 may be adapted for the uptake of only oligomers of xylose of greater length than xylose or xylobiose. The continual presence of adequate supplies of free xylan could allow for such specialization. To test this, we used a syrup of acid-hydrolyzed xylan, that contains oligomers of many different chain lengths, as a substrate. No growth occurred, however, this may be due to inhibitory products produced during acid hydrolysis of the xylan. An unrelated, cellulolytic, thermophilic anaerobe that also utilizes a restricted range of substrates has recently been described (Hudson et al. 1990).

An additional special characteristic of strain B1 is its source of isolation. Previously, *Dictyoglomus*-like organisms have only been isolated from natural thermophilic environments; geothermal hot springs, both alkaline and acid. Strain B1 is the first isolate from a man-made source, a site that is also well removed from any natural thermophilic site. *Dictyoglomus*-like organisms are relatively rarely found as the major organisms in enrichment cultures with fermentable carbohydrates (Patel et al. 1987; Saiki et al. 1985). The factors which determine the presence of *Dictyoglomus*-like organisms in a hot-spring or determine whether they will occur as the major organism in enrichment cultures remain unknown. Xylan is the major component of plant hemicelluloses and is second only to cellulose as the most abundant biopolymer in nature (Puls and Putanen 1989). The wood of some trees is up to 50% xylan and significant concentrations of xylans are present in the wastewaters from the paper pulping industry (Biely 1985). Such waste waters and other sources are, after application of xylanases to facilitate their bioconversion, abundant potential substrates available for the commercial applications (Biely 1985; Wong et al. 1988). Stable and thermoactive xylanases are desirable for such processes as this permits enzyme recycling and easier handling and storage (Wong et al. 1988). Xylanases from thermophilic microorganisms, such as strain B1, may be important catalysts in future biotechnological utilization of plant xylans.

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