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Purification and characterization of thermostable pectate-lyases from a newly isolated thermophilic bacterium, *Thermoanaerobacter italicus* sp. nov.

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Abstract A novel thermophilic spore-forming anaerobic microorganism (strain Ab9) able to grow on citrus pectin and polygalacturonic acid (pectate) was isolated from a thermal spa in Italy. The newly isolated strain grows optimally at 70°C with a growth rate of 0.23 h^{-1} with pectin and 0.12 h^{-1} with pectate as substrates. Xylan, starch, and glycogen are also utilized as carbon sources and thermoactive xylanolytic (highest activity at 70°–75°C), amylolytic as well as pullulolytic enzymes (highest activity at 80°–85°C) are formed. Two thermoactive pectate lyases were isolated from the supernatant of a 300-l culture of isolate Ab9 after growth on citrus pectin. The two enzymes (lyases **a** and **b**) were purified to homogeneity by ammonium sulfate treatment, anion exchange chromatography, hydrophobic chromatography and finally by preparative gel electrophoresis. After sodium dodecylsulfate (SDS) gel electrophoresis, lyase **a** appeared as a single polypeptide with a molecular mass of 135 000 Da whereas lyase **b** consisted of two subunits with molecular masses of 93 000 Da and 158 000 Da. Both enzymes displayed similar catalytic properties with optimal activity at pH 9.0 and 80°C. The enzymes were very stable at 70°C and at 80°C with a half-life of more than 60 min. The maximal activity of the purified lyases was observed with orange pectate (100%) and pectate-sodium salt (90%), whereas pectin was attacked to a much lesser extent (50%). The K_m values of both lyases for pectate and citrus pectin were 0.5 g·l^{-1} and 5.0 g·l^{-1}, respectively. After incubation with polygalacturonic acid, mono-, di-, and trigalacturonate were detected as final products. A 2.5-fold increase of activity was obtained when pectate lyases were incubated in the presence of 1 mM Ca^{2+}. The addition of 1 mM ethylenediaminetetraacetic acid (EDTA) resulted in complete inhibition of the enzymes. These heat-stable enzymes represent the first pectate-lyases isolated and characterized from a thermophilic anaerobic bacterium. On the basis of the results of the 16S rRNA sequence comparisons and the observed phenotypic differences, we propose strain Ab9 as a new species of *Thermoanaerobacter*, namely *Thermoanaerobacter italicus* sp. nov.

Key words *Thermoanaerobacter italicus* sp. nov. · Thermostable · Pectate lyase

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Introduction

Pectin is basically a branched heteropolysaccharide consisting of a main chain of (1,4)-α-D-polygalacturonate, which is partially methyl esterified. Along the chain, L-rhamnopyranose residues are present which are the binding sites for side chains composed of neutral sugars. This polysaccharide is an important plant material that is present in the middle lamellae as well as in the primary cell walls. Enzymatic pectin degradation is widely applied in food technology processes. In fruit-juice extraction and wine-making processes, pectinolytic enzymes are used to increase juice yield, reduce viscosity, improve color extraction from fruit skins, and to macerate fruit as well as vegetable tissues.

Pectinolytic enzymes can be classified into two main groups, namely the methylesterases which remove the methoxy groups from pectin, and the depolymerases (hydrolases and lyases) which attack both pectin and pectate. Pectinolytic bacteria have been isolated from a wide variety of habitats such as trees, lakes, soil, rumen, mullet gut, and the human intestinal tract. Production of pectin hydrolases by bacteria has not yet been described but these enzymes

have been isolated from fungal cultures (Riou et al. 1992). Pectin lyases, which were discovered by Albersheim et al. (1960), are mostly synthesized by fungi but some endo-acting enzymes have been isolated in *Erwinia* and *Pseudomonas* spp. (Itoh et al. 1982; Kamamiya et al. 1974; Schlemmer et al. 1987; Sone et al. 1988). Pectate lyases are mostly produced by bacteria, require alkaline pH values for optimal activity (pH 8–10) and are strongly dependent on Ca^{2+} (Whitaker 1991).

Despite the large number of studies on pectinolytic enzymes, little attention has been focused on the enzymes from thermophilic bacteria. Degradation of particularly starch and cellulose by thermophilic bacteria and archaea has been largely investigated during the last decade. These enzymes are more stable than the enzymes from their mesophilic counterparts (Antranikian et al. 1996). Pectin degradation by thermophilic bacteria has been reported for *Thermoanaerobacterium thermosulfurigenes* (Schink and Zeikus 1983a), *Clostridium thermocellum* (Spinnler et al. 1986), *Thermoanaerobacter thermohydrosulfuricus* (Wiegel et al. 1979), *Clostridium thermosaccharolyticum* (van Rijssel and Hansen 1989), *Bacillus stearothermophilus* (Karbassi and Vaughn 1980), and *Desulfurococcus amylolyticus* (Bonch-Osmolovskaya et al. 1988). The industrial use of such enzymes at high temperature will be of great value since the contamination risk is reduced, and the solubility of polymeric substrates and the rate of diffusion are increased.

Data on thermostable pectinolytic enzymes from thermophiles, however, are still not sufficient to make proper comparisons with similar enzymes from mesophiles. During the search for novel, thermostable, pectinolytic enzymes, we isolated a new thermophilic anaerobic strain from a thermal spa in Italy. After growth on citrus pectin and pectate, this bacterium produces two thermoactive lyases that have a very high affinity for polygalacturonate. These enzymes were purified to homogeneity and studied in detail. This is the first report on the production of pectate lyases by a thermophilic anaerobic bacterium.

Materials and methods

Collection sites

Water and mud samples were collected from different thermal spas in the north of Italy (Abano Terme, Calzignano Terme, Montegrotto Terme, Battaglia Terme, Sirmione, and Agnano Terme). Temperatures at the sampling sites ranged between 40° and 75°C. Samples were transferred into sterile 50-ml vials that were immediately sealed with butyl-rubber stoppers and kept at 4°C in thermic containers until stored in the laboratory.

Bacteria and culture conditions

Acetomicrobium flavidum (DSM 20664^T), *Clostridium fervidus* (DSM 5463^T), *Clostridium thermolacticum* (DSM 2910^T), *Clostridium thermosaccharolyticum* (DSM 571^T), *Thermoanaerobacterium thermosulfurigenes* (DSM 2229^T, 3896), *Thermoanaerobacter thermohydrosulfuricus* (DSM 567^T), *Thermoanaerobacter ethanolicus* (DSM 2246^T), *Thermoanaerobacter finnii* (DSM 3389^T), *Thermoanaerobacter brockii* (DSM 1457^T), *Thermoanaerobacter acetoethylicus* (DSM 2359^T), *Dictyoglomus thermophilum* (DSM 3960^T), and *Fervidobacterium nodosum* (DSM 5306^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany, and cultivated on media suggested by DSM. For the search for new isolates, 1 or 2 ml of liquid and 5–10 g of solid sample was inoculated into 45 ml of prereduced medium in 150-ml serum vials and the enrichment cultures were incubated anaerobically at 60°, 70°, 80°, and 90°C. KOKO medium of the following composition (amount/liter) was used in morphological and most physiological tests: K_2HPO_4, 1.6 g; NH_4Cl, 0.5 g; $MgSO_4 \cdot 6\ H_2O$, 0.16 g; $NaH_2PO_4 \cdot 2H_2O$, 1 g; $CaCl_2 \cdot 2H_2O$, 0.06 g; tryptone, 1.0 g; yeast extract, 1.0 g; meat peptone, 1.0 g; trace elements solution SL-4 (Balch et al. 1979), 10 ml; vitamin solution (Balch et al. 1979), 10 ml; $NaHCO_3$, 1 g; resazurin, 0.5 mg; substrate, 0.5% (w/v); cysteine hydrochloride, 0.3 g; $Na_2S \cdot 9H_2O$, 0.5 g. The medium was prepared under N_2 following anaerobic techniques as described by Balch and Wolfe (1976). Before autoclaving, the pH was adjusted to 6.8–7.0 with NaOH and the Na_2S was sterilized separately and added just before the inoculum, as well as calcium chloride, bicarbonate, substrates, and cysteine.

The utilization of various substrates was tested using 15-ml Hungate tubes with 10 ml KOKO medium and 0.5% (w/v) of the specific carbon source. Stock solutions of carbon sources were autoclaved separately. Growth was recorded after three days of incubation at 65°C by measuring the optical density (600 nm), pH value, and gas production. In order to determine the optimal growth temperature, the strain was inoculated in 50 ml medium amended with 0.5% (w/v) pectin and incubated at different temperatures (37°–85°C).

Gelatin hydrolysis was tested using KOKO agar plates supplemented with 0.4% (w/v) gelatin. After 4 days, plates were flooded with a saturated solution (at 55°C) of Na_2SO_4 in 1N H_2SO_4. Tests for indole production and nitrate reduction were performed using BBL indole nitrite medium (Becton Dickinson, Cockeysville, MD, USA).

For sporulation, 1% (w/v) (*N*-2-morpholino)propane sulfonic acid (MOPS)-buffered media with 0.2% (w/v) xylose was used (Schink and Zeikus 1983b). Reduction of thiosulfate to elemental sulfur or sulfide was tested in the medium of Schink and Zeikus (1983b) and in KOKO medium to which 0.5% (w/v) glucose, 5 g/l of sodium thiosulfate (filter sterilized), and 0.2 g/l of $FeSO_4 \cdot 7H_2O$ (autoclaved under N_2) were added.

The isolation of pure cultures from the enrichments was carried out by repeated subculturing at 70°C and by isolation of single colonies on agar plates, using the already described medium amended with 2% agar, 0.3% pectin, and 10% pectin-purpur (Rinderknecht et al. 1967). All plating procedures were carried out in an anaerobic glove box

(Andover, England). Colonies showing a clear halo after 2–3 days of incubation were transferred into 5 ml culture medium in Hungate tubes. To ensure the purity of the strains isolated, these were restreaked on agar plates and repeatedly isolated after microscopic observation with respect to cell morphology and colony phenotype. The screening of the isolates for the production of pectin-degrading enzymes was carried out either in 2-l bottles or in a 20-l glass fermentor (BCC, Göttingen, Germany). For the large-scale cultivation of isolate Ab9, cultivation was performed in a 300-l stainless-steel fermentor (Braun, Melsungen, Germany) at 70°C, using N_2 as a gas atmosphere. Continuous cultivation experiments were carried out in a 1-l bioreactor (Minibior 1, BCC) equipped with both pH and temperature control probes. The inoculum size was 10% and all experiments were conducted at 70°C, with agitation speed of 10–200 rpm and under anoxic conditions (N_2). A phase-contrast microscope (Zeiss Axioplan Jena, Germany) was used for routine examinations and to obtain microphotographs of bacteria.

Characterization of isolate Ab9

The identification of isolate Ab9 was based on morphological as well as physiological tests. In order to determine the optimal growth temperature, the strain was inoculated in 50 ml complex medium amended with 0.5% (w/v) pectin and incubated at different temperatures (37°–85°C). The utilization of various substrates was tested in 10 ml complex medium with 0.5% (w/v) of the specific carbon source. After 24 h, the optical density (600 nm) and pH values were determined. The G+C DNA content was determined by HPLC according to Mesbah et al. (1989) after extraction and purification of DNA (Johnson 1985), and DNA-DNA hybridizations were performed by the spectrophotometric method of DeLey et al. (1970).

Genomic DNA extraction, polymerase chain reaction (PCR)-mediated amplification of the 16S rRNA gene, and purification of the PCR products were as described previously (Rainey et al. 1992). Double-stranded PCR products were directly sequenced using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the protocol provided by the manufacturer. Sequencing primers used in this study were those described by Stackebrandt and Charfreitag (1990). The purified sequence reactions were subjected to electrophoresis using the Applied Biosystems 373 DNA sequencer.

Pectin and xylan degradation

The utiliation of pectin or xylan as carbon sources by the isolated strains was tested by measuring the residual amount of substrate after growth. Hydrolysis of the residual substrate was carried out by adding 50 µl of 5 M H_2SO_4 to 500 µl of substrate solution (0.1%–0.2% w/v) followed by incubation at 100°C for 3 h. After neutralization by the addition of 50 µl 10 M NaOH, the amount of reducing sugar released was determined by the dinitrosalicylic method (Bernfeld 1955). Standard curves were obtained after hydrolysis of the polymers (0%–0.2% w/v) resuspended in complex medium.

Fermentation products

H_2 and CO_2 were determined by a gas chromatograph (Varian, Walnut Creek, CA, USA) which was equipped with a Porapack Q (80/100 mesh) column (Supelco, Bellefonte, PA, USA) and a thermal conductivity detector. Volatile fatty acids and alcohols were analyzed using a Hewlett-Packard 5880A Gas Chromatograph (Hewlett Packard Avondale, PA, USA) equipped with a Chromosorb WAW 10% sp-100 glass column (Supelco) and a flame ionization detector. Acetate and lactate were determined by enzymatic assays (catalog no. 148261 and 139084, Boehringer, Mannheim, Germany).

Enzyme assays

Enzymatic tests were carried out after centrifugation of cultures (12 000 × *g*, 40 min) and concentration up to 100-fold using the Amicon system mod. 8400 and 8050 (Amicon, Lexington, MA, USA). Amylolytic and pullulytic activities were determined in supernatants as previously described (Miyazaki 1991; Nasser et al. 1976). Xylanolytic activities were determined with cell suspensions by incubation of 200 µl xylan solution (0.5% w/v in 50 mM Tris/HCl pH 8.0) and 50 µl enzyme solution at 70°C for 5–60 min. After clarification of samples by centrifugation, the reduced sugar released was determined (Bernfeld 1955) using xylose as a standard. One unit of activity was defined as the amount of enzyme required to produce 1 µmol of xylose/min. The activity of pectate-degrading enzymes was measured by following the increase of the reducing sugar. The reaction mixture contained 100 µl of pectate solution (0.5% w/v in 50 mM Tris/HCl pH 8.0, 1.5 mM $CaCl_2$) and 10–25 µl enzyme sample. The incubation was carried out at 70°C for 5–60 min. Reducing sugars were assayed with neocuproine (Stephens et al. 1974); samples (0.1 ml) were added to 1 ml reagent A (26.5 g Na_2CO_3 per liter) which inactivated the enzyme. After the addition of 0.5 ml reagent B (400 mg neocuproine and 200 mg $CuSO_4 \cdot 5H_2O$ per liter), samples were incubated at 100°C for 15 min, then cooled on ice and the extinction measured at 450 nm. D-galacturonic acid (0–0.15 µmol) was used as a standard and one unit of activity was defined as 1 µmol of reducing sugar liberated per min.

Pectate lyase activity was determined spectrophotometrically at 230 nm (Collmer et al. 1988). Sample blanks were used to correct for nonenzymatic release of the reduced sugar. To determine the optimum pH of the enzyme, the same protocols were used except for the substitution of reaction buffers with 0.12 M Universal buffer (Britten and Robinson 1931) to obtain values from pH 4.0 to pH 12.0. The temperature optimum was determined by performing the appropriate assays at the temperature indicated. The effect of temperature on the enzyme thermostability was investigated in supernatants after incubation at different

temperatures. In all cases, the incubation was carried out in closed Hungate tubes in order to prevent boiling of the solution and, after various time intervals, samples were withdrawn, clarified by centrifugation and enzymatic activities determined. The protein content of enzyme samples was determined using bovine serum albumin as a standard (Lowry et al. 1951). The same procedure was used to screen all anaerobic strains isolated (2-l cultures) as well as the isolate Ab9 after cultivation in the 20 or 300-l fermentor.

Enzyme purification

After growth of isolate Ab9 on citrus-pectin (0.5% w/v), the culture broth (300l) containing extracellular enzymes was centrifuged at 12000 × *g* for 40 min. After concentration of the supernatant to 1.2l by a Sartocon II system (Sartorius) equipped with an Ultrasart Model (20000 cut-off), solid ammonium sulfate was added to the supernatant solution up to 40% saturation. The precipitate formed was collected by centrifugation, dissolved in 20 mM Tris/HCl pH 7.5, and dialysed overnight against the same buffer. The same procedure was repeated to reach an ammonium sulfate saturation of 80%. The dialysed enzyme solution (275 ml) was then used as a crude enzyme preparation. All procedures were carried out at 4°C.

Step 1. The sample was applied to a 5 × 40 cm Q-Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden) pre-equilibrated with 20 mM Tris/HCl buffer, pH 7.5 at 4°C. After washing extensively with the same buffer, proteins were eluted by a NaCl gradient (0–500 mM in 20 mM Tris/HCl buffer pH 7.5, 2 × 1000 ml) at a flow rate of 5 ml/min and fractions of 11 ml were collected. The eluted enzyme fractions that showed activities on citrus pectin were concentrated 14 times (up to 70 ml) under nitrogen pressure in a 10-ml Amicon chamber with a 10000 cut-off membrane (Amicon), and dialyzed against the same buffer.

Step 2. The enzyme preparation from Step 1 was dialyzed against 20 mM sodium phosphate (pH 7.0, 1.2 M ammonium sulfate) and applied to an Octyl-Sepharose column (2.6 × 32 cm) pre-equilibrated with the same buffer. The column was then washed extensively with the same buffer and the enzyme finally eluted with sodium phosphate buffer only (without ammonium sulfate). Fractions of 2–3 ml were collected at a flow rate of 5 cm/h and the active enzyme fractions were pooled, concentrated, and dialyzed.

Step 3. The sample from Step 2 was further dialyzed against 20 mM sodium phosphate buffer (pH 7.0, containing 1.2 M ammonium sulfate) and then applied to preparative gel electrophoresis (see gel electrophoretic methods). For determination of the molecular mass of proteins the following standard proteins were used: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa), and ovalbumin (45 kDa).

Characterization of hydrolysis products

Analysis of the products from the enzymatic hydrolysis of pectate was performed by thin-layer liquid chromatography as described by Lund and Brocklehurst (1978) after incubation of 0.5 U of purified pectate-lyases with pectate (0.25%) for 24 h at 70°C. Enzyme samples were dialyzed overnight at 4°C in 20 mM sodium phosphate buffer pH 7.0 and incubated at 70°C with 0.5% (w/v) starch or pullulan for 0, 2, 4, 12, 24, 48, and 72 h. After incubation, samples were clarified by centrifugation and sugars analyzed by separation on an HPLC Aminex HPX-42A column (Bio-rad, Richmond, CA, USA).

Gel electrophoresis

The protein pattern of extracellular samples was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10.0%; SDS-PAGE) after boiling the samples for 3–5 min as described by Laemmli (1970). Silver staining of protein bands was carried out according to Heukeshoven and Dernik (1985). Activity staining was performed on native gels (Karbassi and Luh 1979), polyacrylamide gradient gels (5%–27.5%), and SDS gels. Protein bands possessing pectinolytic activities were detected after incubation of the gel for 15 min at 70°C with an overlay gel (7% acrylamide, 0.05% polygalacturonic acid (PGA), 1.5 mM $CaCl_2$, and 0.13% tetramethylethylenediamine). The overlay gel was then incubated at room temperature for 5 min in a 0.1-M iodoacetamide solution and again in a triphenyltetrazoliumchloride solution (0.1% in 0.5 M NaOH) at 100°C until red bands appeared.

The final purification step of the enzymes was performed by preparative gel electrophoresis. Concentrated fractions from Octyl-Sepharose chromatography (950 µl with 0.9 mg protein, 50 U pectate-lyase) were mixed with 100 ml buffer solution (10 mM sodium phosphate pH 7.0, 0.1% bromophenol blue, 5% crystalline sucrose), and applied to an acrylamide gradient gel (5%–10%) in a Bio-rad Prep Cell system model 491 (Bio-rad, Hercules, CA, USA). Elution of the protein was carried out at 4°C in Tris-glycine buffer (pH 8.0) at a constant voltage of 150 V and collecting fractions of 900 µl for 15 h.

Results

Microorganisms degrading pectin

Attempts were made to screen for microorganisms capable of growth on citrus pectin (0.5%) anaerobically between 60° and 70°C. From 29 strains investigated, 4 strains, namely *Clostridium thermolacticum* (DSM 2910^T), *Clostridium thermosaccharolyticum* (DSM 571^T), and *Thermoanaerobacterium thermosulfurigenes* (DSM 2229^T and DSM 3896), degraded approximately 50% of the substrate, and the maximal optical density at 578 nm after 18 h of cultivation

was between 0.5 and 0.9. The major fermentation products from pectin were ethanol (5–14 mM) and acetate (8–19 mM). *C. thermosaccharolyticum* formed in addition trace amounts of butyrate and butanol. Growth of *Fervidobacterium nodosum*, *Thermoanaerobacter acetoethylicus, Thermoanaerobacter finnii, Thermoanaerobacter acetobutylicus*, and *Thermoanaerobacter brockii* on pectin was poor ($OD_{578\,nm}$ 0.2–0.6). Pectin was not utilized by *Acetomicrobium flavidum, Clostridium fervidus*, or *Dictyoglomus thermophilus.* Due to the inability of the screened microorganisms to grow efficiently on pectin, enrichment experiments on pectin were performed. From the experiments at 70°C on a complex medium and on 0.5% citrus pectin, a number of pure cultures were obtained after repeated cultivations and plating on pectin-purpur agar plates. No growth was observed in the enrichments at 80° or 90°C. A total of 14 strains were investigated and, after growth on citrus pectin, optical density, reducing sugars, and organic acids were determined. Four isolates grew very well on pectin (OD $\geq$ 0.7) and one strain (Ab9) was able to degrade up to 80% of the substrate (maximum OD 0.85). All other isolates were able to degrade only a limited amount (30%) of pectin. Ethanol appeared as the main fermentation product but acetate and lactate were also detected. The new isolate was also able to grow on starch, xylan, and lactate. Due to the ability of strain Ab9 to degrade pectin efficiently, it was chosen for further investigations.

Characterization of isolate Ab9

Cells of strain Ab9 were rod-shaped, measuring 0.4–0.75 × 2–6 μm. Chains up to 50 μm in length could be observed during growth in KOKO medium with glucose (Fig. 1a). Motility was never detected. Cells stained Gram-negative. Spherical, terminally located spores were produced in MOPS-buffered media (Schink and Zeikus 1983b) with 0.2% (w/v) xylose as the substrate (Fig. 1b). Colonies on KOKO glucose agar were 2–3 mm in diameter, round with an entire margin, greyish-white, opaque, and with a glossy surface.

The optimum temperature for growth was 70°C and growth occurred between 45°C and 78°C. The pH optimum was around 7.0. NaCl up to 1% (w/v) did not affect growth. Growth was totally inhibited by 10 μg/ml of the antibiotics cephalosporin, erythromycin, kanamycin, or rifampicin.

Strain Ab9 was capable of fermenting a large number of sugars (amygdalin, arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannose, melezitose, melibiose, raffinose, sucrose, trehalose, and xylose) as well as polymers such as starch, glycogen, inulin, pectin, and xylan. Cellulose was not utilized and gelatine was not hydrolyzed. Preferred substrates (OD $\geq$ 1.0) were arabinose, fructose, galactose, glucose, xylose, D-glucosamine, lactose, maltose, saccharose, and pectin.

During growth on glucose or other fermentable sugars, the pH of the KOKO medium fell from 6.8 to 4.7–5.0, and optical densities (600 nm) of $\geq$1.0 were reached. The growth rate using glucose as substrate was 0.33 h^{-1} and the doubling time (t_d) was 2.1 h. If glucose was replaced by pectin the growth rate and the t_d were 0.23 h^{-1} and 3 h, respectively. The products formed after 15 h of growth on 0.5% (w/v) glucose were ethanol (26.6 mM), lactate

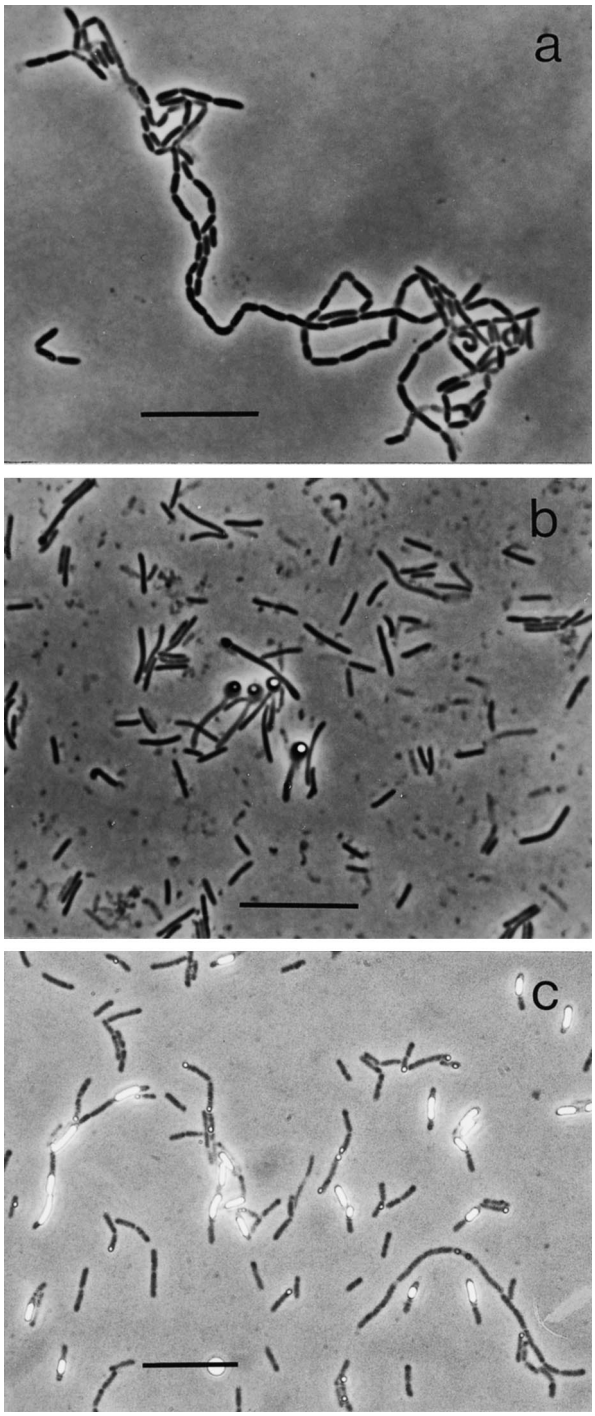

Fig. 1. Phase-contrast microphotographs of strain Ab9: **a** chain-forming cells from a 24-h culture in KOKO medium with glucose; **b** sporulating cells from 48-h culture in MOPS-buffered KOKO medium with 0.2% xylose; **c** elemental sulfur deposits in thiosulfate-supplemented medium. *Bar*, 10 μm

(12.4 mM), acetate (2.2 mM), succinate (0.3 mM), H_2, and CO_2.

Strain Ab9 was able to reduce thiosulfate to elemental sulfur. Massive sulfur formation occurred after 24 h of growth in the 20-mM-thiosulfate-containing medium of Schink and Zeikus (1983b) (Fig. 1c). If KOKO medium was supplemented with both thiosulfate and 2 g/l of ferrous sulfate, the medium turned black soon after growth became visible, and this was followed by sulfur formation. The blackening of the medium indicated that strain Ab9 was capable of reduction of thiosulfate to sulfide, which precipitated as ferrous sulfide, in addition to the reduction to elemental sulfur.

DNA base composition and DNA–DNA homology

The DNA mol% G+C content was 34.4 as determined by HPLC. The levels of DNA–DNA homology between strain Ab9 and *Thermoanaerobacter thermohydrosulfuricus* DSM 570 and *Thermoanaerobacterium thermosulfurigens* DSM 2229 were 57% and 30%, respectively.

Phylogenetic analysis of strain Ab9

The almost complete 16S rDNA sequence of strain Ab9 was manually aligned with published sequences of members of the *Bacillus*/*Clostridium* subphylum of the Gram-positive bacteria. Pairwise evolutionary distances were computed using the correction of Jukes and Cantor (1969) and used in the construction of a phylogenetic dendrogram using the least squares distance method of De Soete (1983). The phylogenetic position of strain Ab9 within the radiation of the thermophilic clostridia is shown in Fig. 2. Strain Ab9 clusters with *Clostridium thermocopriae* having 97.0% sequence similarity. The sequence similarity of strain Ab9 to the *Thermoanaerobacter* species and *Acetigenium kivui* which comprise this subcluster is in the range of 93.5%–95.8% (Table 1).

Determination of enzyme activities

After growth on starch, both amylolytic and pullulytic activities were detected in culture supernatants of strain Ab9 as well as in cell suspensions. A total of 510 U/l of amylolytic activity and 670 U/l of pullulytic activity were detected in the cell suspensions. A significant proportion of these enzymatic activities appeared to be cell-bound (69% and 31%, respectively). The optimal temperature for both enzyme activities was 80°–85°C. At 50°C, only 10% of both activities was measurable and at 90°C, 50% of the amylolytic and 90% of the pullulytic activity were still detected. The optimum pH was 5.5; at pH values of 4.0 and 8.0, 20% of the pullulytic activity and 40% of the amylolytic activity were measured.

The analysis of starch, pullulan, and amylose degradation by HPLC showed the presence of α-amylase, pullulanase (type II), and α-glucosidase or glucoamylase. After growth of the cells on 0.5% xylan at 70°C for 15 h, 60% of the substrate was consumed. Using oat-spelt xylan as substrate, the temperature and pH of the xylanolytic enzyme was determined. Optimal activity was at 75°C and pH 5.5. At 90°C and 40°C, 10% of the xylanolytic activity was present: 20% of the activity was detected at pH 4.0 and pH 8.5. After growth of the isolate Ab9 on citrus pectin for

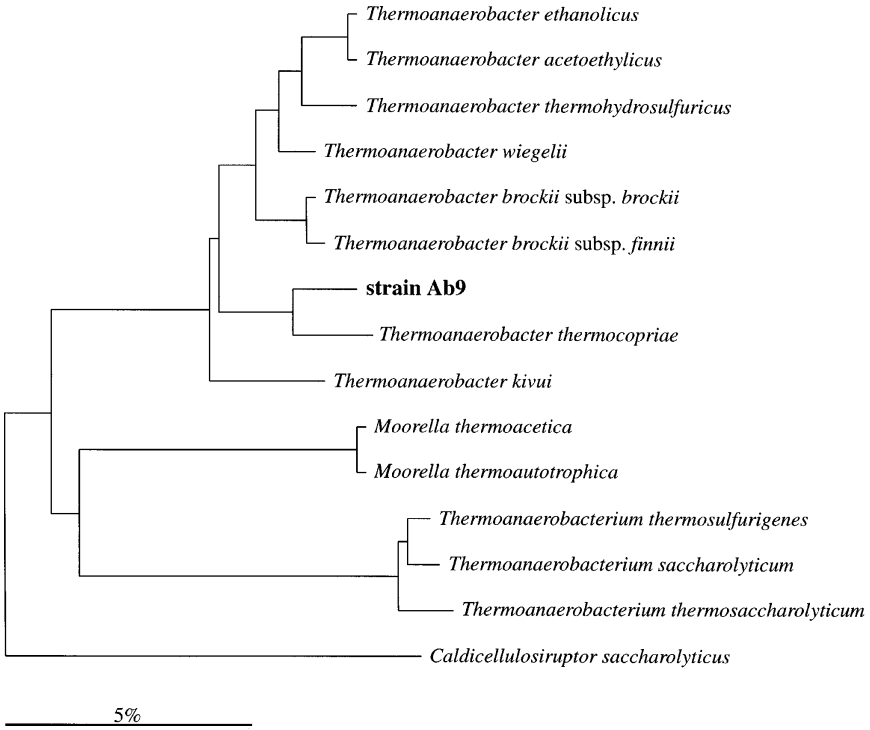

Fig. 2. Unrooted phylogenetic dendrogram indicating the phylogenetic position of strain Ab9 within the radiation of the thermophilic clostridia. The *scale bar* indicates 5 inferred substitutions per 100 nucleotides

Table 1. 16S rDNA similarity values between strain Ab9 and related taxa

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Strain Ab9	–														
2. *T. thermocopriae*	97.1	–													
3. *T. thermohydrosulfuricus*	94.6	94.1	–												
4. *T. ethanolicus*	93.9	94.1	97.8	–											
5. *T. acetoethylicus*	93.8	94.0	97.4	99.3	–										
6. *T. wiegelii*	95.1	95.0	97.2	98.2	97.8	–									
7. *T. brockii* subsp. *brockii*	95.8	95.0	96.8	96.3	96.6	97.4	–								
8. *T. brockii* subsp. *finnii*	95.6	94.8	96.6	96.0	96.3	97.2	99.3	–							
9. *T. kivui*	94.7	94.3	95.4	94.9	94.9	96.1	96.3	96.0	–						
10. *Tb. thermosaccharolyticum*	86.9	86.5	87.3	87.0	86.9	87.8	87.2	86.9	87.9	–					
11. *Tb. saccharolyticum*	87.0	86.3	87.2	87.0	87.0	87.3	87.3	86.9	88.1	97.8	–				
12. *Tb. thermosulfurigenes*	87.4	86.7	88.0	87.8	87.7	88.2	87.7	87.4	88.3	97.9	98.5	–			
13. *M. thermoacetica*	88.5	88.3	88.4	88.4	88.4	89.2	89.1	88.9	89.0	87.8	87.4	87.8	–		
14. *M. thermoautotrophica*	88.2	87.8	87.9	87.9	87.9	88.7	88.6	88.4	88.7	87.5	87.3	87.6	99.1	–	
15. *C. saccharolyticus*	85.8	86.1	85.0	85.1	85.3	86.3	85.8	85.6	85.8	84.1	83.9	84.3	84.5	84.4	–

T., *Thermoanaerobacter*; *Tb.*, *Thermoanaerobacterium*; *M.*, *Moorella*; *C.*, *Caldicellulosiruptor.*

12–24 h at 70°C, the neocuprein test of reducing sugars showed only a very limited substrate degradation. Better results were obtained after growing the isolate on pectate under the same conditions. The pectinolytic activity had a temperature range of 40°–85°C with an optimum at 75°C; at 60° and 78°C, 50% of the enzymatic activity was detected. The pH range for the pectinolytic activity was 4.8–10.6 with an optimum at pH 8.9; at pH values of 7.0 and 9.7, 50% of the activity was still measurable. The alkaline pH optimum for the enzyme activity suggested that lyases and not hydrolases could be involved. Both pectin and pectate lyases are in fact known to be active at high pH values, often requiring Ca^{2+} ions. These enzymes break the glycosidic bonds between galacturonic acid residues via β-elimination, resulting in an unsaturated product with a double bond between C_4 and C_5 (Whitaker 1984); this reaction can be quantified by photometric analysis at 232 nm (Collmer et al. 1988). The involvement of pectin/pectate lyases was documented by an increase of absorbance at 232 nm, as well as the increase of activity in the presence of Ca^{2+} ions and of the optimal activity at alkaline pH values.

Physiological experiments

Batch cultivation of isolate Ab9 was carried out at 70°C in a 20-l glass fermentor with citrus pectin or pectate as carbon source and gassing with N_2. The highest cell yields were obtained after 14 h and 16 h with pectin (OD 0.85) and pectate (OD 0.6), respectively. After 16 h of cultivation the substrate concentration (pectin, pectate) decreased from 0.5%–0.6% to 0.1%. The fermentation products formed were acetate, ethanol, lactate, H_2, and CO_2. Figure 3 shows the fermentation time-course of strain Ab9 on pectin. After 16 h of growth, about 110 U/l of pectate lyase was formed and approximately 50% of the enzyme was released into the medium. Similar results were obtained when pectate was used as substrate.

In continuous culture experiments, the effect of dilution rate and substrates (pectate, pectin, glucose) on growth and enzyme production was also investigated. The highest

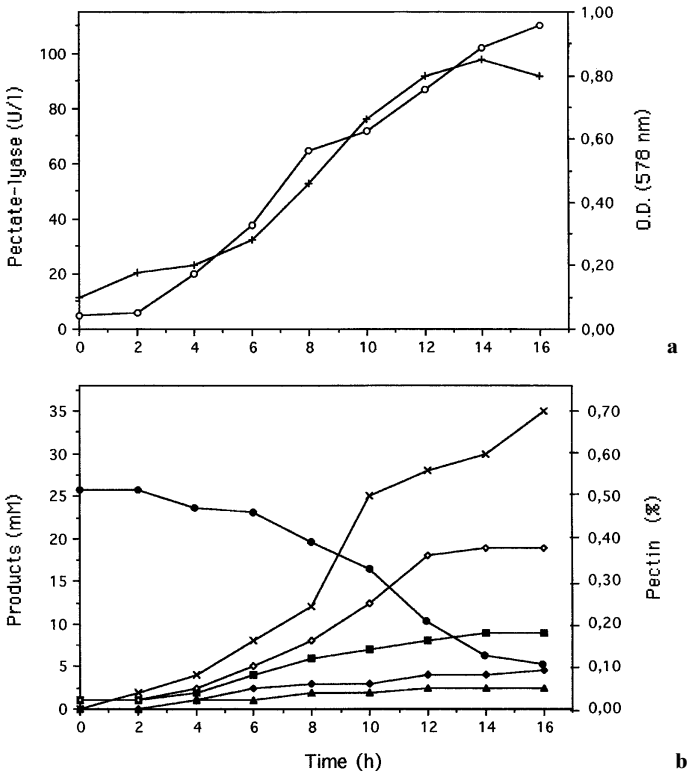

Fig. 3. Fermentation time-course of strain Ab9 cultivated at 70°C in complex mineral medium containing pectin as the main carbon source. The degradation of the substrate, the production of metabolic products, optical density, and pectate-lyase activity were monitored at different time intervals. **a** +, OD (578 nm); *open circles*, pectate-lyase; **b** *closed circles*, pectin; ×, CO_2; *open diamonds*, ethanol; *squares*, acetate; *closed diamonds*, H_2; *triangles*, lactate

amount of pectate lyase activity (200 U/l) was obtained with 0.2% (w/v) pectin at a dilution rate of 0.08 h^{-1}. Under these conditions, the chemostat was run under substrate limitation. Increase of pectin up to 1.0% (w/v) caused an increase in the OD from 0.2 to 0.75 but a decrease in pectate lyase concentration (from 200 U/l to 150 U/l). Under all these conditions, 75% of the enzyme was cell-bound. The acetate

concentration increased from 5 mM to 20 mM and the ethanol concentration from 2 mM to 10 mM. Further experiments in continuous culture were performed with pectate or glucose instead of pectin. Under steady state conditions at a dilution rate of 0.08 h^{-1}, 620 U/l and 200 U/l of pectate lyase was detected if pectate and pectin were used as substrates, respectively. In the presence of glucose only 10 U/l were formed.

Purification of pectate lyases

The supernatant of a 300-l culture of strain Ab9 cultivated on citrus pectin was concentrated up to 1.2 l. The first purification step was an ammonium sulfate precipitation (Table 2). Further purification was achieved by anion-exchange chromatography and hydrophobic chromatography. After this step, two enzyme peaks with a similar specific activity (55 U/mg) were eluted. After the preparative gel electrophoresis (PGE) of the fraction showing the highest activity, two separate pectate-lyases (PGE **a** and PGE **b**) with specific activities of 57 and 102 U/mg, respectively, were eluted.

Gel electrophoresis

Analysis of the PGE **a** sample by native gradient gel electrophoresis showed one protein band with a molecular mass of 148 000 Da, whereas the PGE b sample showed two bands with a molecular masses of 200 000 Da and 252 000 Da. After SDS-PAGE of PGE **a,** only one protein band with a molecular mass of 135 000 Da was detected. Treating the PGE **b** sample with SDS resulted in the detection of two protein bands with molecular masses of 93 000 Da and 158 000 Da. Activity staining was possible only in native gradient gels and not in SDS gels, even after incubation in a 0.5% Triton X-100 solution.

Characterization of pectate lyases

The two pectate lyases (**a**, **b**) showed identical catalytic properties. The temperature for activity of both enzymes ranged between 40° and 95°C with an optimum at 80°C (Fig. 4a). The pH range for activity was between 5.0 and 11.0; the pH optimum for the activity of pectate-lyases was about 8.0 (Fig. 4b). The enzymes were very stable at 70°C and at 80°C; the half-life of both enzymes was about 60 min (Fig. 4c). Kinetic properties of the purified pectate lyases were investigated with pectate-sodium salt, orange pectate, citrus pectin, and orange pectin. The highest affinity constant (K_m) values were obtained with pectate-sodium salt (0.05% which is equivalent to 0.05 mM pectate) and citrus pectin (0.5% which is equivalent to 0.16 mM pectin). Like other lyases, the purified enzymes from isolate Ab9 were highly stimulated by the presence of Ca^{2+} ions. A 2.5-fold increase in activity was obtained when the pectate lyases were incubated with 1 mM of Ca^{2+}, and a slight increase in activity (125%) was observed after the addition of Mn^{2+}; Mg^{2+} and Co^{2+} had no influence on the enzyme activity. The

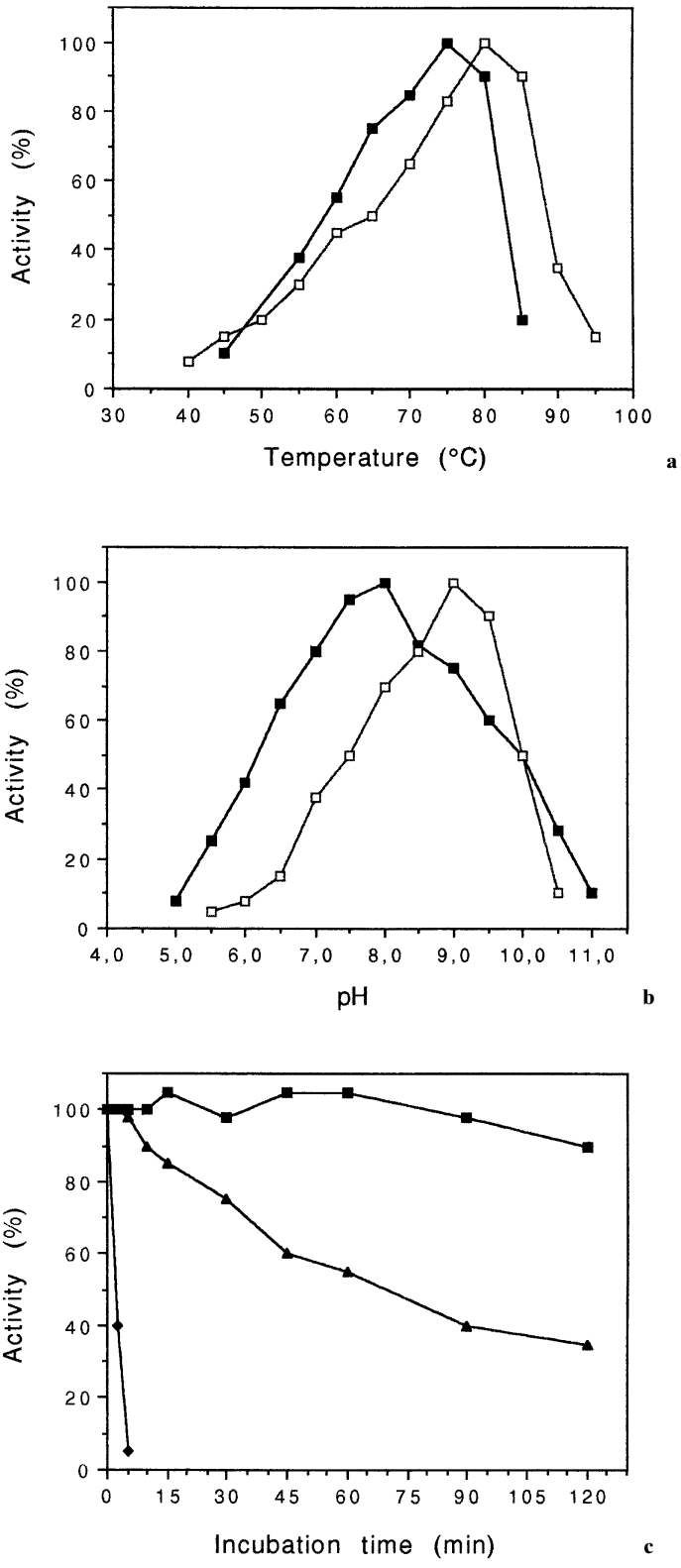

Fig. 4. Effect of temperature (**a**) and pH (**b**) on the pectate lyase activity from isolate Ab9. The relative pectate lyase activity of the purified enzymes **a** and **b** (*open squares*) and pectate lyase in the culture supernatant (*closed squares*) was determined at various temperatures and pH values. **c** Thermostability of the purified enzyme (pectate lyases a and b) determined after incubation at 70°C (*squares*), 80°C (*triangles*), and 90°C (*diamonds*)

Table 2. Purification of pectate lyases from *Thermoanaerobacter italicus*

Step[a]	Fraction volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	1220	5636	4636	0.8	100	–
P40	2035	1610	4233	1.0	91	1.3
P80	275	778	1911	2.4	41	3.0
Q-Sepharose	72	75	1122	15	24	18
Octyl-Sepharose	20	5.0	263	55	6	65
PGE **a**	11	0.5	27	57	0.6	68
PGE **b**	24	0.55	55	102	1.2	121

Cells were cultivated at 70°C in a 300-l fermentor and the culture broth was concentrated up to 1220 ml.
[a] P40 and P80, ammonium sulfate precipitation (40% and 80% saturation); Q-Sepharose, anion-exchange chromatography; Octyl-Sepharose, hydrophobic chromatography; PGE, preparative gel electrophoresis; **a**, **b**, active fractions from PGE.

addition of 1 mM ethylenediaminetetraacetic acid (EDTA) completely inhibited the purified pectate-lyases. No inhibition, however, was observed if 1 mM of Ca^{2+} was already present in the sample.

Discussion

The pectinolytic strain Ab9 is a strictly anaerobic spore-forming thermophile that is able to reduce thiosulfate to elemental sulfur. Accumulation of sulfur in the medium and on the cells resulting from thiosulfate reduction has been described as a characteristic property of members of the genus *Thermoanaerobacterium* (Lee et al. 1993). However, the results of 16S rDNA sequence analysis of strain Ab9 indicate that it is unrelated to the genus *Thermoanaerobacterium*, and falls within the radiation of the genus *Thermoanaerobacter* and related taxa (Fig. 2). The *Thermoanaerobacter* cluster, designated cluster A by Rainey et al. (1993), had a intracluster similarity range of 95.2%–99.5%. The transfer of *Acetigenium kivui* and *Clostridium thermocopriae* to the genus *Thermoanaerobacter* has been proposed by Collins et al. (1994). The phylogenetic relatedness of strain Ab9 to this *Thermoanaerobacter* cluster would indicate its allocation to this taxon.

Phenotypically, strain Ab9 is unlike the currently accepted species of *Thermoanaerobacter* and *Acetigenium kivui* in the combination of several properties such as spore production (positive for Ab9), Gram staining reaction, motility, gelatin hydrolysis, and butyrate production (all negative for Ab9). Furthermore, a large number of sugar polymers were utilized, and the reduction of thiosulfate to sulfur has not been reported in any of the species of the phylogenetic cluster A.

Strain Ab9 is most closely related (97.0% sequence similarity) to the cellulolytic *Clostridium thermocopriae*. However, despite several phenotypic similarities, strain Ab9 differs from *C. thermocopriae*, for instance, in that it has a 10°C higher temperature optimum for growth; an inability to utilize cellulose; growth on mannitol, melezitose, and melibiose; and does not form butyric acid as a fermentation product.

On the basis of the results of the 16S rDNA sequence comparisons and the observed phenotypic differences we propose strain Ab9 as a new species of *Thermoanaerobacter*, namely *Thermoanaerobacter italicus*: Kozianowski et al. (sp. nov.) i.ta.li.cus. L.n. *italia* Italy; M.L. masc.adj. *italicus* pertaining to Italy, where the organism was isolated. The characteristics of the new species are as for strain Ab9 summarized in the Results section and in Table 3. The type strain, Ab9, isolated from medicinal mud (fango) of Abano Terme, Italy, was deposited as DSM 9252 in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

The new isolate was able to grow on citrus pectin (80% degradation) with a doubling time of 3 h. On the same substrate, *C. thermosaccharolyticum* and *C. thermocellum* had doubling times of 1.5 and 4.2 h, respectively (Spinnler et al. 1996; van Rijssel et al. 1993). As previously reported for *C. thermosaccharolyticum* (van Rijssel et al. 1993), pectin degradation was always incomplete due to the heterogeneous structure of the polymer itself which is characterized by the presence of many alternative residues (arabinose, galactose, rhamnose, xylose, fucose, and glucose) unevenly distributed over the pectin chain.

In batch cultures of strain Ab9 cultivated on citrus pectin, a total of 110 U/l was produced and a large amount of enzyme (40%–50%) appeared to be cell-bound. Enzyme activity tests clearly showed that pectate and not pectin was the favorite substrate although higher cell yields were usually obtained with pectin as a carbon source. The largest enzyme production (620 U/l) was obtained in continuous culture with 0.5% pectate at a flow rate of $0.08\,h^{-1}$. In the presence of pectin, lower enzyme yields (30%) were obtained under the same culture conditions. The results obtained with glucose (10 U/l) clearly suggest that pectate lyase production is repressed by the monomeric substrate glucose.

Purification of the pectinolytic enzymes to homogeneity was achieved in four steps. An initial attempt to precipitate the enzymes with ethanol was unsuccessful due to the simultaneous precipitation of the remaining substrate. Only by the gradual addition of ammonium sulfate (up to 80% saturation) to the concentrated culture supernatant, was it possible to recover about 41% of the pectinolytic activity.

Table 3. Distinguishing properties among *Thermoanaerobacter italicus* and species of *Thermoanaerobacter*, *Acetogenium*, and *Clostridium*[a]

Properties	*Thermoanaerobacter italicus*	*Acetogenium kivui*	*Clostridium thermocopriae*	*Thermoanaerobacter*				
				acetoethylicus	*brockii*	*ethanolicus*	*finnii*	*thermohydrosulfuricus*
Spores	+	−	+	−	+	−	+	+
Gram-staining	−	−	−	−	+	+/−	V	V
Cell wall peptidoglycan diamino acid	m-DAP	nr	nr	nr	nr	m-DAP	m-DAP	m-DAP
Motility	−	−	−	+	+	+	+	nr
DNA mol% G+C	34.4 (HPLC)	38 (Bd)	37.2 (Tm)	31 (Tm)	30–31.1 (Tm)	32 (Tm)	32 (Tm)	29–32 (Tm)
Gelatin hydrolysis	−	nr	−	nr	nr	+	nr	−
Growth temperature:								
optimum	70°C	66°C	60°C	65°C	68°C	69°C	65°C	67°–69°C
range	45°–80°C	50°–75°C	47°–74°C	40°–80°C	35°–80°C	37°–78°C	40°–75°C	37°–78°C
Growth on $H_2 + CO_2$	nd	+	nr	nr	nr	nr	nr	nr
Utilization of polymers:								
cellulose	−	−	+w	−	−	−	nr	−
glycogen	+	nr	+w	nr	nr	nr	nr	nd
pectin	+	−	−	−	−	+	nr	+
inulin	+	nr	−	nr	nr	nr	nr	−
starch	+	−	+w	+	+	+	nr	+
xylan	+	nr	+w	−	+	+	nr	+
Fermentation products	ethanol lactate acetate (succinate)	acetate	ethanol butyrate acetate (lactate)	acetate ethanol (butyrate) (iso-butyrate)	ethanol acetate lactate	ethanol acetate lactate	ethanol acetate lactate	ethanol acetate lactate (butyrate) (iso-valerate) (iso-caproate)
Thiosulfate reduction								
to S°	+	nr	nr	nr	−	−	nr	−
to sulfide	+	nr	nr	+	+	+	nr	+

[a] Data from original descriptions and from Wiegel et al. (1979).
nr, not reported; nd, not determined; V, variable; +, positive; −, negative; +w, weakly positive; m-DAP, meso-diamino pimelic acid; Bd, Buoyant density; Tm, Thermal melting point.

Similar yields of recovery have been reported for the pectate lyases of *Bacillus pumilus* and from a thermophilic *Bacillus* strain (Dave and Vaughn 1971; Karbassi and Luh 1979). After hydrophobic chromatography (Octyl-Sepharose), two sets of fractions (I and II) with high pectinolytic activities were obtained. Both samples gave two bands (**a** and **b**) after preparative gel electrophoresis but no differences in catalytic activities were found after the four enzyme preparations were tested. The isolated enzymes were more active on pectate than on pectin, and were characterized by high absorbance at 232 nm. Based on these results, both pectinolytic enzymes **a** and **b** were classified as pectate lyases (Collmer et al. 1988; Rexova-Benkova and Markovic 1976). This is the first report on the presence of this type of enzyme in anaerobic thermophilic bacteria. Thermostable pectinolytic enzymes, were purified from *T. thermosulfurigenes* and *C. thermosaccharolyticum* but they were described as pectate hydrolases and pectate methylesterases (Schink and Zeikus 1983a; van Rijssel and Hansen 1989). The addition of Ca^{2+} highly stimulated the action of both pectate lyases but it was not absolutely required. This is in accordance with the results previously reported for other pectin/pectate lyases, although the role of calcium ions is not yet well established (Albersheim et al. 1960). The K_m values obtained in the presence of pectin (0.5%) and pectate (0.05%), with both lyases **a** and **b**, are similar to the values reported for other thermoactive pectate lyases (Dave and Vaughn 1971; Karbassi and Luh 1979; Miyazaki 1991; Nasser et al. 1976; Yoshida et al. 1991). Only the pectate-lyase isolated from *Thermomonospora fusca* was reported to have a much higher substrate affinity (K_m value 0.002%) at the optimal temperature of 60°C (Stutzenberger 1987).

With a temperature optimum for activity at 80°C, the pectate lyases from isolate Ab9 represent the most thermoactive pectinolytic enzymes ever described. At 70°C, no loss of activity was observed after 2 h, and at 80°C, the half-life of both lyases was 60 min. According to the gel electrophoretic experiments, pectate lyase **a** is a single polypeptide chain with a molecular mass of 135 000 Da whereas pectate lyase **b** consists of two subunits with molecular masses of 93 000 Da and 158 000 Da. This suggests that the purified lyases either represent multiple forms of the same enzyme or are the result of proteolysis during the enzyme secretion process. The possibility that the three lyases are associated in a high molecular weight enzymatic complex cannot be excluded; similar structures have been described in *C. multifermentans* (Sheiman et al. 1976) and *C. thermosaccharolyticum* (van Rijssel and Hansen 1989).

The use of thermostable pectin-degrading enzymes for many applications in the food industry is attractive but the isolation of new thermostable pectinolytic enzymes is necessary to expand the range of potential applications with anaerobic thermophilic bacteria. New insights are also particularly expected from similar investigations with hyperthermophilic microorganisms that are able to produce enzymes with new catalytic properties.

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