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The Endopolysaccharide Metabolism of the Hyperthermophilic Archeon *Thermococcus hydrothermalis*: Polymer Structure and Biosynthesis

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Abstract The endopolysaccharide accumulated by *Thermococcus hydrothermalis* was extracted and purified from a 4 h culture. It presented an "amylopectin-like" structure with an average chain length of 14 and a ramification degree of 7.5%. The glucosyltransferase was isolated, partially purified and characterized. The molecular mass was 42 kDa by SDS PAGE and 85 ± 5 kDa by gel filtration. This enzyme was able to use both Uridine-5′-DiPhosphoGlucose (UDPG) and Adenosine-5′-DiPhosphoGlucose (ADPG) as substrates. Optimal pH and temperature for the enzyme were 5.5 and 80°C, respectively. In the presence of 3.2 mM ADPG, the half life of the protein was 6 min at 110°C. The apparent K_m value with the two substrates was 0.9 mM, but the V_{max} was 9.7 fold higher for ADPG. A branching activity was also detected at high temperature, up to 80°C by different methods: phosphorylase stimulation, iodine, and branching linkage assays.

The biosynthesis of reserve endopolyssacharides such as starch and glycogen represents a metabolism common to many living organisms [7, 21, 22]. Starch, for most of the higher and lower photosynthetic organisms, is a highly structured macromolecule containing two types of polysaccharide: amylopectin (α-1, 4-D-glucan, presenting approximately 5% of alpha-1,6 linkages) and amylose (minor fraction, moderately ramified) [21]. Glycogen, the second common endopolymer, is produced by most of bacteria and non photosynthetic Eukarya and is less structurated than starch but highly ramified (8–10%) [26].

The building of these macromolecules depends exclusively on two enzymatic activities: elongation activity (ADP-glucose (or UDP-glucose): α-1,4-D-glucan-α-4-glucosyltransferase EC 2.4.1.21) and branching activity (α-1,4-D-glucan: α-1,4-D-glucan-6-glucosyltranferase EC 2.4.1.18). The first one catalyses the glucose transfer from the glucosylnucleotide (ADPG or UDPG) to the non-reducing end of an α-1,4-D-glucan. The second step consists of a cleavage of an α-1,4-D-glucan and its transfer into an alpha-1,6 position. These two steps will implicate various numbers of isoforms as a function of the type of organism [6, 9, 13, 19].

Degradative and biosynthesis enzymes are simultaneously produced [23]. Many papers have been published on polysaccharides degrading hyperthermophilic enzymes [3, 10, 15, 16]. However, until now, few studies have been concerned with the biosynthesis of the storage carbohydrates in the *Archaea* Kingdom [12]. We now report the nature and structure of the polysaccharide accumulated by the Euryarcheota *Thermococcus hydrothermalis* [11]. Primary characterization of the glucosyltransferase and branching activities is also presented.

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Material and Methods

Bacterial strain and culture conditions. *T. hydrothermalis* AL662^T (CNCMI-1319) [11] was cultivated anaerobically as previously described [15] at 85°C in a fermentor using BS medium (pH 6.0) supplemented with 0.4% (wt/vol) maltose instead of starch.

Extraction of the intracellular content. After a 4 h period, the cells were harvested by centrifugation and resuspended in 200 mM phosphate buffer (pH 6.0), containing 0.01% NaN_3. The concentrated suspension was submitted to a French Press (52.2 MPa) and finally

centrifuged for 3 h at 10000 g, at 4°C. The supernatant containing the intracellular content was stored at 4°C.

Analysis of the endopolysaccharide accumulated by *Thermococcus hydrothermalis*. *Quantification*. Polysaccharide was quantified using enzymatic assay kit for starch determination (Diffchamb).
Purification. For 1 g of freeze-dried intracellular content, 10 ml of a NaOH 6 N solution containing 2% SDS (wt/vol) was added, and then incubated for 12 h at 100°C to hydrolyse most of the proteic fraction. The solution was then neutralized by a 20% (vol/vol) acetic acid solution, and ultrafiltrated against distilled water 0.02% NaN_3 with a 10 kDa Millipore cellulose membrane. A second proteolyse was carried out on the freeze-dried retentat by using 20 ml anhydrous hydrazine. The mixture was incubated for 12 h at 90°C. Residual hydrazine was evaporated under vacuum. The vapors were condensed and collected on 5% sulfuric acid solution and the remaining vapors were trapped through bleach. The solution (neutralized by a 10% aqueous acetic acid solution) was ultrafiltrated as previously described. Protamine sulfate was added (2 mg/ml^{-1}) to the retentat. The mixture, stored for 2 h at 4°C under constant slow stirring, was centrifuged for 1 h at 45000 *g*. The supernatant was ultrafiltrated and the polysaccharide was finally isolated by adding 4 volumes of ethanol (95%). The solution, stored for 12 h at 4°C, was centrifuged at 10000 g for 45 min at 4°C. The supernatant was removed and the pellet was resuspended in distilled water with 0.01% NaN_3. The solution was stored at −20°C.

NMR spectra. The purified fraction was freeze-dried and exchanged twice with 500 μl of Sigma ultra-pure deuterium oxide (99.96%). The freeze-dried sample was dissolved into 99.9% deuterated DMSO (Sigma)/deuterium oxide (5:1) and incubated for 1 h at 80°C before analysis. Proton NMR spectroscopy measurements were recordered at 80°C on a 500 MHz DRX500 Bruker spectrometer in 5 mm tubes (polymer concentration: 2 mg/ml^{-1}). Proton spectra were obtained with 1072 scans per experiment, relaxation delay of 2 between scans. Deuterated DMSO was used as an internal standard reference at 2.65 ppm.

Debranching analysis. Four units of isoamylase (Hayashibara biochemical laboratories) were added to 1 mg of polymer dissolved in 50 mM acetate buffer pH 3.5. The mixture was incubated for 12 h at 45°C. After boiling, centrifugation at 10000 g, and filtration through 0.22 μm Amicon membrane, the debranched glucans were subjected to high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) using a Carbo-Pac PA-100 column (Dionex), flow: 1 ml/min^{-1}, buffer A: 100 mM NaOH, buffer B; 100 mM NaOH/100 mM sodium acetate, linear gradient: from 50 mM acetate to 350 mM in 35 min.

Enzymatic preparation. After ultrafiltration through 10 kDa membrane, 40 mg of intracellular proteins were subjected to a DEAE-Sephadex HR5.5 column (Amersham-Pharmacia Inc.) equilibrated with 20 mM TRIS-HCI buffer pH 8.8. Elution was carried out at 25°C with a discontinue NaCl gradient (from 0 to 1 M added to the former buffer), 1 ml/min^{-1} flow, eluted fraction: 1 ml. The fractions presenting the elongation activity were detected by zymograms and pooled (total protein: 8.8 mg) to be eluted with the same anion exchange chromatography in the same experimental conditions, but with longer salt gradients.

The molecular mass of the native enzyme was estimated by gel filtration with a Sephacryl-S200 16/60 column equilibrated with 100 mM phosphate pH 6.0, 0.01% NaN_3. Sigma standard proteins (low and high molecular weight kits) were used for the column calibration. Enzymatic activity was detected by zymograms.

Electrophoretic techniques. Denaturated polycrylamide gels (30:1, 7.5%, or 10%, 1.5 mm thick) were made using Miniprotean II electrophoretic chamber (Biorad). Proteins (60 to 100 μg) were denaturated by addition of 1.5% SDS (wt/vol) and 4% 2-mercaptoethanol (vol/vol) and incubation for 20 min at 100°C before electrophresis (150 V for 90 min).

Elongation activity was detected by denaturating zymograms containing glycogen 0.3% (wt/vol). After migration, proteins were renaturated in a 40 mM TRIS-HCI buffer, pH 7.5 (4 × 1 h). The elongation of incorporated glycogen was obtained during a 12 h incubation at 85°C in a 50 mM Citrate Phosphate buffer pH 6.0 containing 3.2 mM of cold ADP-glucose or UDP-glucose as previously described [8]. The activity was located and detected in gels by a iodine solution (2.0% KI and 0.2%I_2) [1].

The branching activity was detected following the phosphorylase stimulation method [19]. This technique requires using commercial phosphorylase in order to synthesise short MaltoOligoSaccharides (MOS) from glucose-1-phosphate (GIP). The former sugars were then used as substrates for the branching enzyme assay. This enzyme creates linkages between the MOS's reducing end and the carbon 6 of other MOS glucose residues. The formation of branched polysaccharides led to a high proportion of non-reducing extremities. The presence of non-reducing extremities allowed the phosphorylase to degrade the newly formed oligosaccharides, and to form GIP, allowing the cycle to restart. After proteins migration and renaturation, the gel (containing no glycogen) was incubated at 55°C for 24 h in a 50 mM acetate buffer pH 6.0 with 45 mM Sigma GIP and phosphorylase a (1 mg/ml^{-1}). Iodine solution allowed the branching activity to be monitored. Control experiments were performed to confirm that the activity was not caused by α-1,4-D-glucanotranferase or endogenous phosphorylases.

Protein electroelution. After electrophoresis, proteins were electroeluted using the Biorad Whole Gel Eluter. Electroelution was performed at 200 mA for 30 min, with the migration buffer described before. The harvested samples were dialyzed 6 h at 20°C against 50 mM TRIS-HCI, pH 7.5.

Sample preparation for the isolation of the branching activity. Intracellular content was incubated at 80°C for 30 min with an equal volume of soluble starch 1%. The polysaccharide fraction was precipitated twice with 4 volumes of ethanol 95%, centrifuged at 10000 g for 30 min at 4°C. The co-precipitated proteins were extracted, twice, with a solution containing 2% SDS (wt/vol) and 5% 2-mercaptoethanol (vol/vol). After 10 min at 100°C, the solution was finally centrifuged for 30 min at 10000 g. The supernatant, containing extracted proteins, could be used directly in the electrophoresis experiments. The absence of proteins in the commercial soluble starch was also checked.

Enzyme assays. Protein was measured by the Bradford method (Bio-Rad kit). Elongation activity (20 μl of semi-pure fraction) was assayed at 85°C for 25 min by using 80 μl citrate-phosphate buffer (0.1 M, pH 5.5) containing 2.66 μM ADPG [^{14}C] (25 $\mu Ci/ml^{-1}$; 200 $mCi/Mmol^{-1}$) or 9.2 μM UDPG [^{3}H] (1 mCi/ml^{-1}; 100 $Ci/Mmol^{-1}$) as already described [8]. The pH for the optimal activity was measured at 85°C between pH 4.0 and pH 7.5 using 0.1 M citrate-phosphate and TRIS-HCI buffers. The optimal temperature was determined between 25°C and 110°C with 0.1 M citrate-phosphate buffer pH 5.5. Thermostability was estimated in optimal conditions after enzyme pre-incubation with or without substrate between 80°C and 110°C. Kinetics was studied in optimal conditions using 2.3 μM radiolabelled substrate and various concentrations of cold one. Branching activity was detected after incubation of 0.55% amylose (80 μl) with 40 μl of electroeluted samples at 80°C for 12 h. Branching activity was then detected by the branching linkage and iodine assays [19]. The first method, based on the detection of the reducing sugars liberated after action of commer-

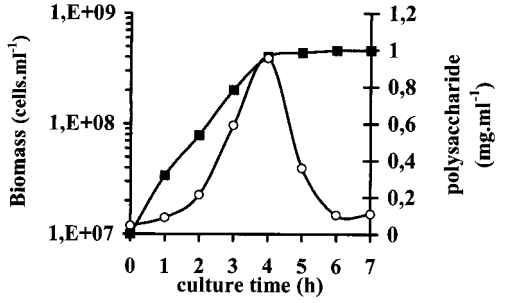

Fig. 1. *Thermococcus hydrothermalis* growth and endopolysaccharide production at 85°C in a 7 l fermentor using BS medium pH 6.0. ■ Biomass, ○ endopolysaccharide.

cial isoamylase was carried out on amylose, which had been ramified by the branching enzyme [25]. The iodine assay consists in the measurement of the iodine-glucan complex absorbency decrease, using 10 μl of sample and 10 μl of the iodine solution, which had been diluted in 980 μl of distilled water. The maximum absorbency frequency, or λmax, is characteristic of each type of polysaccharide (amylose, amylopectin, glycogen, or starch).

Results and Discussion

Cells growth and endopolysaccharide production. Though many culture media have been tested, no minimal media has been found yet for the endopolysaccharide production in the strain *T. hydrothermalis*. Nevertheless, the BS medium, already described as one of the most efficient for enzyme production [14], supplemented with 4 g/l^{-1} maltose led to a sufficient biomass allowing the detection and extraction of an endopolysaccharide. The production profile for the biomass and the endopolymer is shown in the Fig. 1. The strain exhibited a generation time of 40 min. The endopolysaccharide production increased during the exponential growth phase, and showed a maximal accumulation at 4 h of culture, corresponding to the end of this phase (until 1.1 mg of polysaccharide/ml of intracellular content). However the reserve macromolecules were rapidly degraded as soon as the stationary phase started. Although most eubacterial species accumulate glycogen during the stationary phase, there are examples of some eubacterial and archaeal species that synthesize it during the log phase [5, 12]. It was suggested that the decrease of exogenous carbon sources and the need to detoxify some metabolic end-products could lead these micro-organisms to use their glucose stores [24].

Polysaccharide purification and characterization. The polysaccharide purification methods already described were not transposable to the strain *T. hydrothermalis*. König et al. (1982) have noted that the technique based on a separation of the polymer by ClCs gradient with

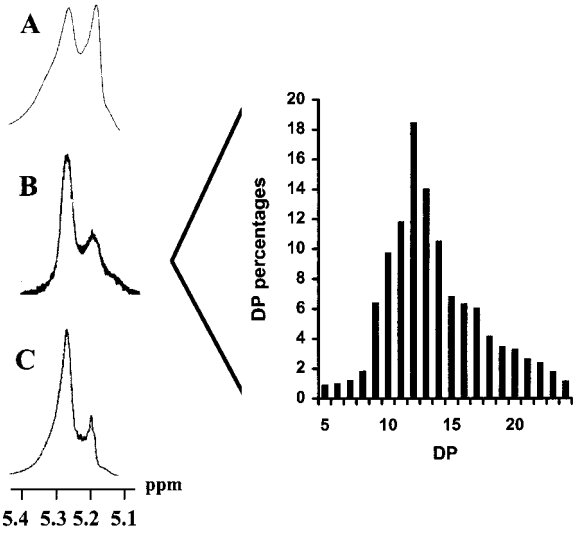

Fig. 2. ^{1}H-NMR analysis of various endopolysaccharides. **A:** *Oyster* glycogen. **B:** *T. hydrothermalis* endopolysaccharide from a 4 h culture. **C:** *Waxy* cultivar Amylopectin. Analysis of the chains length repartition of the archeal polymer (right) by HPAE-PAD using a Carbo-Pac PA-100 column (Dionex).

Sulfolobus acidocaldarius was not applicable to a Euryarcheota strain of the genera Thermoplasmale [12]. We have been confronted with the same problem trying to purify the *T. hydrothermalis* endopolysaccharide. Moreover, the methodology used for mesophilic microorganisms, based on proteins denaturation by boiling [17] was not useful for thermostable proteins of this archaeal strain. For these reasons, we had to design a new purification protocol, leading to a 17% (wt/wt) endopolysaccharide final yield from crude intracellular extracts.

The analysis of the purified polysaccharide fraction by Gas Phase Chromatography after methanolysis has only shown the presence of glucose (data not shown).

NMR spectrum of the purified endopolysaccaride was compared to the amylopectin and glycogen ones (Fig. 2). The area of the two peaks at 5.2 and 5.3 ppm, characteristic of protons linked to the carbon 1 of glucose residues, reveals the relative proportions of long and shorts glucanic chains [2, 4, 20]. The polysaccharide produced by *T. hydrothermalis* showed a 5.3 ppm peak higher than the one observed at 5.2 ppm as in amylopectin, which posseses longer glucan chains than glycogen. The anomeric protons of the glucose residues linked in C6 position as corresponding to the branching points, were visualized around 4.9 ppm. The integration of the peak corresponding to these former protons led to a branching rate estimated at 7.5%. This percentage is a little higher than the one found for amylopectin (5.5% to 6%). The structural characterizations were completed by the analysis of the chain length repartition after enzy-

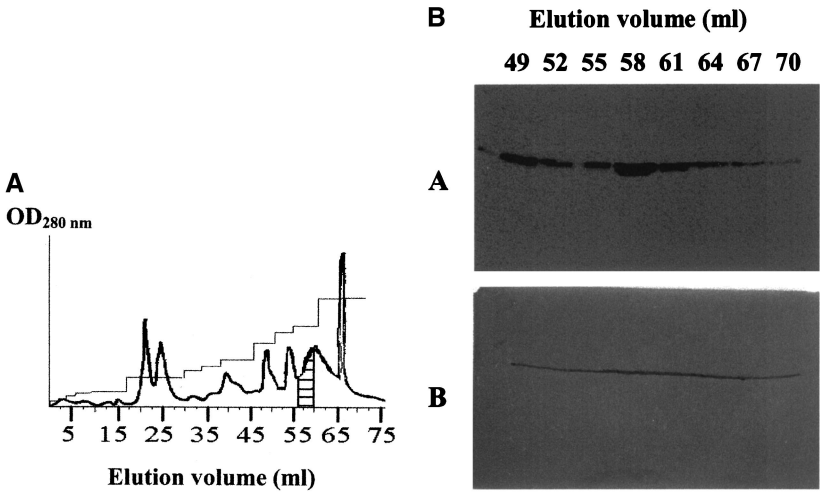

Fig. 3. Partial purification of the glucosyltransferase. **A:** Elution profile after the second anion exchange chromatography. Striped part of the elution profile corresponds to the fractions presenting the maximal glucosyltransferase activity. **B:** Glucosyltransferase zymograms after the second anion exchange chromatography: ADPG and UDPG were used as substrate in zymogram **A** and **B**, respectively. Only active fractions (100 μl) are displayed.

matic debranching (Fig. 2). This histogram displays the prevalence of long chains ($12 < DP < 14$) compared to short chains and thus recalls a structural organization closer to amylopectin ($8 < DP < 20$ depending on the different species) than to glycogen ($5 < DP < 7$) [18]. At the present time, this "amylopectin-like" structure is a unique case in the prokaryotic domain. Nevertheless, it is fundamental to stress that no systematic study of endopolysaccharide structure has been carried out in the different physiological states of the Archaea. So, it is conceivable that this kind of studies, from various culture times, could reveal "glycogen-like" structures as observed with *S. acidocaldarius* [12].

Enzymatic studies—elongation activity. The detection by zymograms and the characterization of the elongation activity were carried out after two anions exchange chromatography steps (Fig. 3) in order to eliminate all the endoploysaccharide degradation activities (pullulanase, alpha-amylase), which are antagonist to the glucosyl transferase. Only the eluted fractions from 57 to 59 ml were used for the glucosyltransferase characterization (protein concentration: 1.2 mg/ml^{-1}). The purification factor was twenty-five fold. The elongation activity was found through one band on denaturing zymograms, with both ADPG and UDPG, indicating the presence of only one protein. The molecular mass of the protein was calculated to be 42 kDa using SDS-PAGE and the molecular mass of the native glucosyltransferase was estimated to be 85 ± 5kDa by gel filtration chromatography, suggesting that the enzyme might be constituted of two subunits.

Physicochemical approaches showed an optimal activity at pH 5.5. This enzyme is active in a broad range pH, 50% of activity remaining between pH 5.2 and pH 7.5. The glucosyltransferase activity is undetectable at 25°C and was found optimal at 80°C. Fifty-five percent of activity remained at 70°C and at 90°C. The enzyme was found inactive at 110°C. These results have to be compared with the ones carried out on *S. acidocaldarius* glucosyltransferase activity which was optimal at pH 7.5 and between 70°C and 80°C [12]. The enzyme thermostability incubated with or without substrate, was assayed between 80°C and 110°C. The glucosyl transferase activity was thermostable at 80°C for at least 2 h and presented a half-life time of 26 min at 90°C, and 3 min at 100°C. In the presence of 3.2 mM ADPG, the half-lives increased to 52 min, 9.5 min, and 6 min at 90°C, 100°C, and 110°C, respectively.

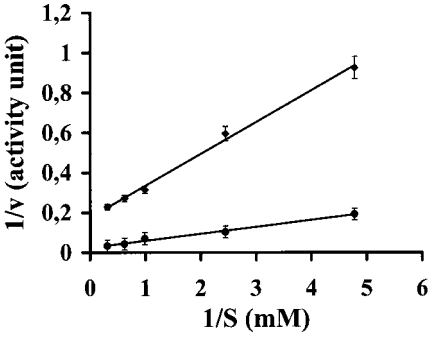

Fig. 4. Kinetics of the glucosyltransferase activity. The synthase activity was assayed at pH 5.5 and at 80°C. One unit is defined as one nmole of ADPG(UDPG) incorporated into glycogen per mg of proteins per min.

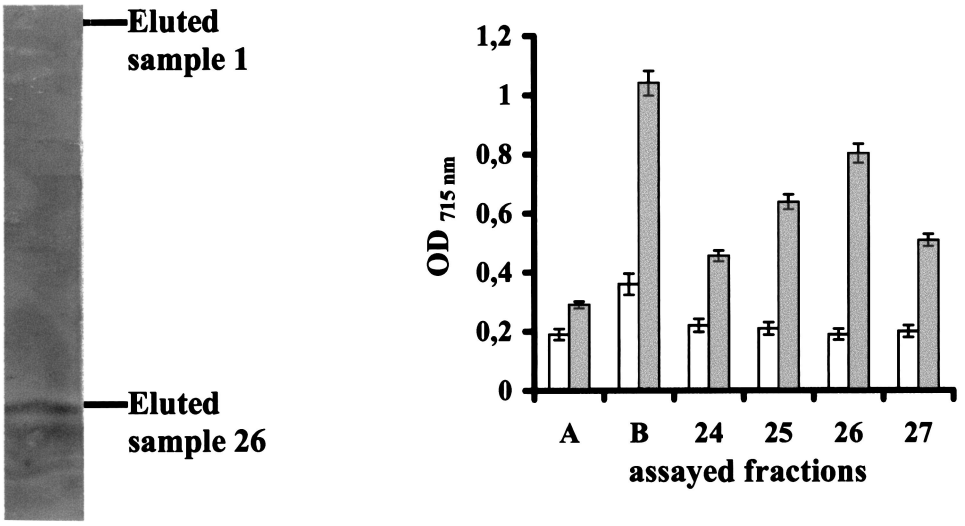

Fig. 5. Isolation of the branching activity. Branching enzyme zymogram (left): Samples (150 μl) were deposed on PAGE-SDS. Branching linkage assays (right) at 80°C: A—Amylose 0.55% (wt/vol). B—Glycogen 0.55% (wt/vol). Samples nt 24 to 27 correspond to electroeluted fractions.

Kinetic experiments revealed that affinities for ADPG and UDPG were the same (apparent K_M = 0.9 mM), but the V_{max} value was 9.7 fold less important for UDPG, suggesting that ADPG was probably the physiological substrate of the enzyme (Fig 4).

This low enzyme/substrate specificity was also observed by Koenig *et al*. (1982) [12] with *S. acidocaldarius*, but the apparent K_M values were stronger with *T. hydrothermalis*.

Enzymatic studies—Branching activity. The branching activity for hyperthermophiles is more difficulte to exhibit and to characterize than the glucosyl transferase one. Assay methods include the use of mesophilic commercial enzymes, involving assay temperatures which do not take into account the optimal activity temperature of archeal hyperthermophilic enzymes. After enzyme preparation, samples were deposed on SDS-PAGE. In our experimental conditions, the "phosphorylase stimulation" allowed us to visualize one band after iodine revelation (Fig. 5). The location on SDS-PAGE of the branching activity was confirmed after proteins electroelution by iodine and "branching linkage" assay (Fig. 5). When amylose was incubated at 80°C with proteins electroeluted from zymogram band, the resulting glucan-iodine complex displayed a maximal absorbency wavelength at 530 nm as for the amylopectin-iodine complex. The presence of branching linkages on these glucans formed from amylose was confirmed by the detection of reducing sugars after isoamylase incubation.

The molecular mass of this enzyme was calculated to be 65 kDa by SDS-PAGE.

Conclusion. The hyperthermophilic Euryacheota, *Thermococcus hydrothermalis*, accumulates an endopolysaccharide having an "amylopectin-like" structure. The biosynthetic pathway of this macromolecule involves at least one identified thermostable and thermoactive elongation protein and one isolated branching protein. Further investigations are under study in our group to complete the characterization of the enzymatic activities involved in the building of this unique reserve polysaccharide.

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