Cloning of an α-glucosidase gene from *Thermococcus hydrothermalis* by functional complementation of a *Saccharomyces cerevisiae mal11* mutant strain

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Abstract **α-Glucosidase is found in methanogenic and thermophilic archaea and also in eukaryotes and bacteria. The gene encoding the enzyme was cloned from *Thermococcus hydrothermalis* by complementation of a *Saccharomyces cerevisiae* deficiency maltase mutant strain. The gDNA clone isolated encodes an open reading frame corresponding to a protein of 242 amino acids. The protein shows 42% identity to a *Pyrococcus horikoshii* unknown ORF but no similarities were obtained with polysaccharidase sequences.**

Key words: α-Glucosidase; Thermophilic archaeon; *mal11* mutant; *Thermococcus hydrothermalis*; *Saccharomyces cerevisiae*

1. Introduction

Many α-glucosidases (α-D-glucoside glucohydrolase, EC.3.2.1.20) have been reported in animals, plants and microorganisms and extensive studies have been done [1]. In animals and plants, the α-glucosidase hydrolyses α-glucans as well as maltooligosaccharides. In microorganisms, it catalyses the successive liberation of α-glucosyl residues from the non-reducing end of short saccharides containing α→(1-4) and/or α→(1-6) linkages. It has very low affinity to polysaccharides and therefore attacks starch at a very slow rate. This enzyme is widely distributed among aerobic and anaerobic microorganisms, where it can be either extracellular, cell-bound or intracellular [2]. Microbial α-glucosidases are conventionally classified into two types (I and II) on the basis of their substrate specificities. Group I hydrolyses aryl glucosides such as phenyl α-glucopyranoside and *p*-nitrophenyl α-glucopyranoside (α-*p*-NPG) more rapidly than maltooligosaccharides such as maltose or isomaltose. The type II group hydrolyses maltooligosaccharides, unlike the type I group [3]. This protein has been extensively studied at the biochemical and molecular levels in a number of bacteria. In contrast, very little is known in archaea. α-Glucosidase has been described and purified from *Sulfolobus solfataricus* [4], *Sulfolobus shibatae* [5], *Pyrococcus furiosus* [6], *Pyrococcus woesei* [7] and *Thermococcus zilligii* [8], but only one gene from archaea has been characterised in *S. solfataricus* [9]. In archaea, α-glucosidase is monomeric (60 kDa in *T. zilligii*) [8] or multimeric (300 kDa in *S. solfataricus* and *S. shibatae*) [4,5] enzyme.

Thermococcus hydrothermalis AL662 is a coccoid, hyperthermophilic, obligately anaerobic, heterotrophic and sulphur-reducing archaeon isolated from a deep-sea hydrothermal vent [10]. This strain is able to produce several amylolytic enzymes: amylopullulanase, α-amylase and α-glucosidase [11]. The latter is a monomeric intracellular protein of 110 kDa and has been purified [12].

Here, we present the isolation, cloning and sequencing of an α-glucosidase gene from *T. hydrothermalis* by functional complementation of a *S. cerevisiae mal11* mutant.

The deduced amino acids sequence of 242 amino acids shows high similarities with an unknown ORF from *Pyrococcus horikoshii*. The deduced N-terminal sequence is different from the sequenced N-terminus of the purified α-glucosidase from *T. hydrothermalis* [12].

Thus we report here, for the first time, the presence of two types of α-glucosidase enzymes in archaea.

The nucleotide sequence has been submitted to the EMBL Nucleotide Database with accession number AJ132781.

2. Materials and methods

2.1. Strains and plasmids

T. hydrothermalis strain AL662 (CNCMI-1319) was obtained from the IFREMER's collection. *Escherichia coli* DH5α (*SupE44ΔLacU169* (*Φ 80lacZΔN15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used as host for cloning. *S. cerevisiae* TCY70 (*MAL12 MAL13 mal11::LEU3 ura3*) was described by Chow et al. [13] and used for cloning the gene. The shuttle bacterial yeast vector pYME1, containing an expression cassette consisting of the *S. cerevisiae* MAL11 promoter and the ADH3 terminator [14], was used in the construction of the gene library. pSK (Stratagene) was used for subcloning in *E. coli*.

2.2. Media and culture conditions

Strain AL662 was grown anaerobically at 85°C in glass bottles in BHI (brain/heart infusion) cystine medium. The culture medium contained 0.92% BHI, 2.3% NaCl and 0.4% cystine.

Transformed *E. coli* DH5α was grown in Luria-Bertani medium containing ampicillin. The yeast strain TCY70 was grown in YPG medium (1% yeast extract, 1% bacto-tryptone, 1% glucose) at 30°C. The transformed strain was grown in minimal medium: 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA) supplemented with glucose (SD medium) or maltose (SM medium) used at 2% as sole carbon source.

2.3. Transformation procedure

Transformation of yeast was carried out as described by Gietz et al. [15]. *E. coli* was transformed according to the method of Cohen et al. [16].

2.4. Preparation, analysis of DNA and molecular techniques

DNA manipulations were carried out using standard methods [17]. Standard procedures were followed for the preparation of plasmid DNA from *E. coli* cultures [18,19], and from *S. cerevisiae* cultures [20]. DNA from *T. hydrothermalis* was prepared as previously described by Godfroy et al. [10]. Restriction endonuclease digestions

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and DNA ligation were performed according to the recommendations of the manufacturers.

2.5. Cloning of the α-glucosidase gene of T. hydrothermalis

A genomic library of *T. hydrothermalis* AL662 strain was constructed by cloning partially digested *Sau*3A chromosomal DNA. The resulting fragments were separated by 1% agarose gel electrophoresis. The fragments between 0.5 and 15 kb were recovered by electroelution and they were afterwards ligated in pYME1 vector at the *Bam*HI site with the T4 DNA ligase. The ligation mixture was carried out at a DNA/vector ratio of 10/1 and was used to transform *S. cerevisiae* TCY70. The gene bank was plated on SD medium agar plates for 4 days at 30°C. Genomic library screening was performed on SM medium agar plates after using the replicating method. Positive clone screening is based on the production of maltose-degrading enzyme which allows recombinant cells to grow at 30°C. At this temperature, archaeal α-glucosidase shows about 10% of its maximal activity [21]. Selected yeast colonies were picked up, plasmids were extracted as described and used to transform *E. coli* DH5α for restriction analysis and sequencing.

2.6. DNA sequencing

DNA sequencing was performed by Eurogentec (Seraing, Belgium). This DNA sequence was determined from pYME clones using the primer walking method. Sequence analysis was performed on an automated sequencer ABI model 377 (ABI, Perkin Elmer).

2.7. PCR procedure for amplification

The α-glucosidase gene was amplified by PCR using genomic DNA from *T. hydrothermalis* as template and the oligonucleotides CGCGGATCCTA**ATGAAAAGCGAGAG** (sense) and AAACTGCAG**TCATAGGAACGCATGCCT** (antisense) as primers. The hybridisation step was performed at 60°C during 1 min. The sense nucleotide was derived from the 5′ DNA sequence of the α-glucosidase gene around the start codon ATG and contained a *Bam*HI site (the sequence from the gene is in bold). The antisense oligonucleotide was derived from the gene sequence around the stop TGA codon and contained a *Pst*I site (the sequence from the gene is in bold). The coding sequence was cloned into vector pSK. The resulting plasmid pAG1234 was digested by *Xba*I and *Pst*I. The resulting fragment was ligated into pYME1 vector. The obtained pTAG235 was used to transform TCY70 cells.

2.8. Southern analysis

Genomics DNA from hyperthermophilic strains such as *P. furiosus*, *P. abyssi* 549, *P. abyssi* 855 and *T. fumicolans* 557 were obtained from Dr J. Dietrich at IFREMER. *T. celer* strain was obtained from Dr E. Legin, University of Reims.

The *Bam*HI/*Pst*I fragment obtained by PCR was digoxigenin-labelled using hexanucleotide primers and Klenow enzyme as described by the manufacturer (DIG high prime DNA labelling and detection kit, Boehringer Mannheim) and was used as specific probe in Southern blot analysis. Hybridisation was performed overnight at 55°C in 1% blocking solution, 5×SSC, sarcosyl 0.1% and SDS 0.02% followed by two washes in 2×SSC, 0.1% SDS at room temperature and two 15 min washes at 55°C in 0.5×SSC, 0.1% SDS.

2.9. α-Glucosidase activity measurements

α-Glucosidase was extracted in 50 mM citrate-phosphate buffer (pH 5.5), after yeast cell disruption with glass beads. Total α-glucosidase was assayed at 85°C in 50 mM citrate-phosphate buffer (pH 5.5) and 0.5% maltose as substrate.

3. Results and discussion

A DNA expression library constructed from *T. hydrothermalis* DNA was screened via complementation of a non-re-

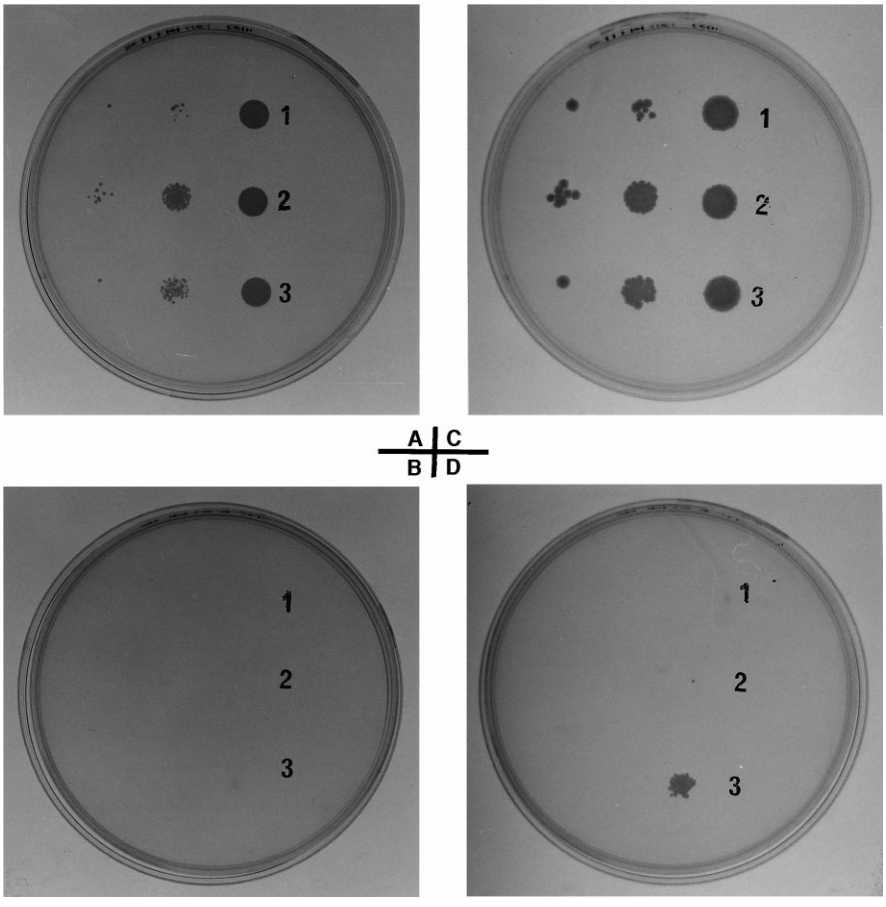

Fig. 1. Complementation of TCY70 *S. cerevisiae* mutant strain with (1) pYME1, (2) p41F2 and (3) pTAG235. Growth after 3 days on SD glucose (A) or SD maltose (B) and after 6 days on SD glucose (C) or SD maltose (D). From left to right, 2×10^7, 2×10^5 and 2×10^3 cells.

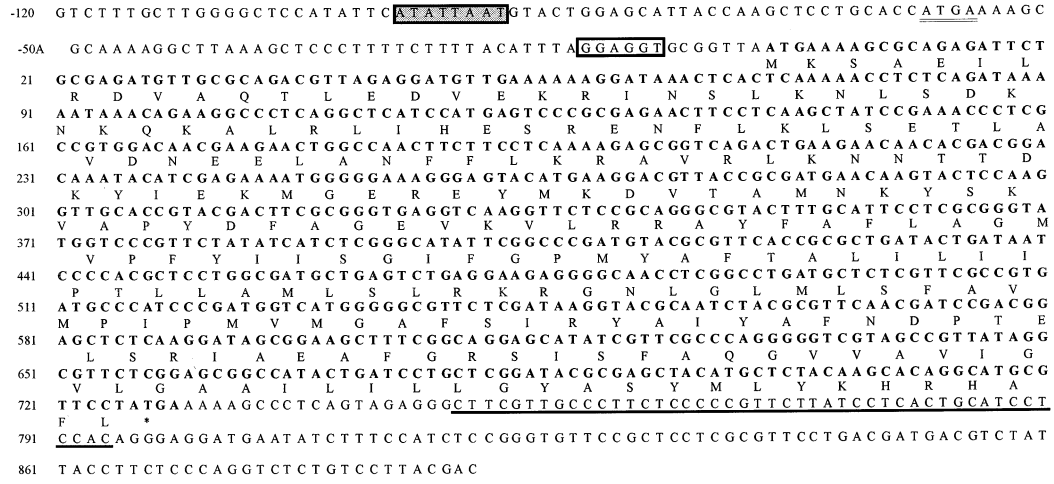

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the α-glucosidase gene from *T. hydrothermalis*. The nucleotide sequence between −120 and +889 sites is represented. The putative ribosome binding site GGAGGT is boxed. The promoter region containing a consensus box A sequence ATATTAAT has a grey background. The putative start of transcription site is underlined. After the TGA stop codon, a pyrimidine-rich sequence is underlined.

verting *S. cerevisiae* TCY70 strain [13]. In yeast, the MAL locus encodes three gene products, one of which is an intracellular α-glucosidase (*MAL1*). A second gene product, maltose permease (*MAL2*), allows transport of maltose in the cell. Both genes are coordinately regulated at the transcriptional level under the influence of a third gene, *MAL3*. In the TCY70 strain, only the *MAL1* gene is inactive. Seventeen colonies grew on maltose from 12 000 screened clones, while the TCY70 strain transformed with pYME1 did not grow.

DNA plasmid analysis of prototrophic strains, able to grow on medium containing maltose as sole carbon source, revealed inserts of different sizes. Comparison of the restriction maps indicated a common sequence. We used the construct p41F2, carrying an approximately 2-kb *Eco*RI/*Pst*I fragment, for further analysis. For confirmation, the p41F2 plasmid was used to retransform the *S. cerevisiae* TCY70 strain. Transformants were plated on SM medium and grew after 6 days of culture.

This 2-kb fragment was sequenced on both strands using primer walking. Only one complete potential ORF (ORF_{242}) was identified in this insert in opposite orientation against the vector promoter.

In order to increase expression by using a strong promoters, the entire ORF_{242} sequence was cloned into the pYME1 vector.

A PCR strategy (see Section 2) was used to amplify the nucleotide sequence corresponding to the ORF_{242} using *T. hydrothermalis* genomic DNA. This amplified product was isolated and inserted into pSK and pYME1 plasmids to obtain respectively pAG1234 and pTAG235.

When a Southern blot prepared with digested genomic DNA from *T. hydrothermalis* was probed with a digoxigenin-labelled *Bam*HI/*Pst*I fragment from pAG1234, a 9.4-kb *Eco*RI fragment, a 11-kb *Bam*HI fragment, two *Sau*3A fragments of 1 and 1.7 kb and two *Hin*dIII fragments of 1.1 and 2.4 kb were found to specifically hybridise the probe. So, the insert used as probe is derived from the chromosome of *T. hydrothermalis*.

In order to prove the function of the isolated gene, the plasmid pTAG235 was used to transform TCY70 competent cells and transformants were plated on SD medium. Transformants were washed with sterile water and plated on two media (SD and SM) at different dilutions (2×10^7, 2×10^5 and 2×10^3 cells) (Fig. 1 and Table 1).

After 3 days of culture, TCY70(pYME1), TCY70(p41F2) and TCY70(pTAG235) grew on SD medium and at all dilutions. On SM medium, no growth was observed. After 6 days, TCY70(pTAG235) and TCY70(p41F2) (one colony) were found to grow on maltose. Expression of the *T. hydrothermalis* DNA in the mutant yeast strain restores its ability to use maltose as a carbon source for growth. According to these results, we deduced that the nucleotide sequence of the ORF cloned in pTAG235 encoded a maltose degrading enzyme, an α-glucosidase encoding gene. The archaeal α-glucosidase gene can complement the yeast *mal11* mutant in the same way as it has previously been shown that the aspartate transcarbamylase gene from *P. abyssi* can complement a *pyrB E. coli* deficient strain [22].

The α-glucosidase activity in crude yeast extract was determined at 85°C, pH 5.5 with maltose used as substrate. No activity was detected in the non-transformed or pYME1

Table 1
Growth of *S. cerevisiae* strains in the different media

	SD medium		SM medium	
	3 days culture	6 days culture	3 days culture	6 days culture
TCY70 (pYME1)	++	+++	−	−
TCY70 (p41F2)	++	+++	−	+
TCY70 (pTAG235)	++	+++	−	++

Plates were incubated at 30°C for 3 or 6 days on SD glucose or SD maltose; −, no growth; + weak growth; ++, good growth; +++, excellent growth.

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ORF242      1 MKSAEILRDVAQTLEDVEKRINSLKNLSDKNKQKALRLIHESRENFLKLSETLAVDNEEL 60
              +KS EILR+V++ L+   +++  +  L +  K+KA++L+ E+ +NFLKLS  + VDN ++
PH1373      4 VKSPEILREVSENLKKASEKLEKVGVLEENKKRKAIKLLTEASQNFLKLSSEVEVDNVQM 63

ORF242     61 ANFFLKRAVRLKNNTTDKYIEKMGEREYMKDVTAMNKYSKVAPYDFAGEVKV-LRRAYFA 119
              A FF KR+V +KNN+TD+ IE++GE+EYMK V  MN YSK A YDF   + + L++ Y
PH1373     64 AEFFRKRSVEIKNNSTDRGIERIGEKEYMKSVEKMNLYSKAAFYDFKRSMLLELKKFYRL 123

ORF242    120 FLAGMVPFYIISGIFG-PMYAFTALILIIPTLLAMLSLRKRGNLGLMLSFAVMPIPMVMG 178
              F+ GM  ++++SG+   P  A TALIL IP +L+MLSL++RG  GLML++AV PIP++
PH1373    124 FIFGMALYFVLSGLSTRPELAITALILAIPAILSMLSLQRRGYTGLMLAYAVSPIPIIQS 183

ORF242    179 AFSIRYAIYAFNDPTELSRIAEAFGRSISFAQGVVAVIGVLGAAILILLGYASYMLYKHR 238
              A  IR       +P  + + AEA G+S  F      ++ +L      LL Y  Y L KHR
PH1373    184 AMLIRMFYSVVTNPEAIRKAAEALGKSQEFVVVYSYLVIILSLIDFGLLSYGLYGLAKHR 243

ORF242    239 HAFL 242
              +AFL
PH1373    244 YAFL 247
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Fig. 3. Amino acid sequence homology of translated sequences from *T. hydrothermalis* α-glucosidase gene (ORF242) and *P. horikoshii* unknown ORF (PH1373). + indicates similarity between two amino acids.

transformed TCY70 strains. Significant activity (8.21 mmol glucose/min/ml of culture) was detected following transformation of the TCY70 mutant with pTAG235 and maltose induction during 48 h. Physico-chemical properties have previously been determined: 85–90°C and pH 5.5 [21].

The TCY70(p41F2) strain was able to growth on maltose indicating that this archaeal promoter is recognised by *S. cerevisiae*, as has been reported by Wettach et al. [23].

The 726-bp open reading frame encodes a single polypeptide of 224 amino acids with a predicted molecular mass of 27 328 Da (Fig. 2). 13 bp upstream of the coding region is the sequence GGAGGT, similar to the putative ribosome binding site already described in archaeal promoters. Moreover, the sequence is complementary to the ACCUCC sequence found in the 3′ end part of the 16S rRNA from *T. hydrothermalis* (GenBank accession number Z70244) [10]. A 'box A' promoter region, ATATTAAT, was also identified between −96 and −89 according to the ATG at the +1 position. As already described for other archaeal genes, a transcription termination pyrimidine-rich sequence is found (20 bp downstream of the α-glucosidase gene stop codon). The G+C content of the *T. hydrothermalis* α-glucosidase gene (53%) is slightly lower than that of the entire organism's genome (58%) [10]. The calculated molecular mass (27.3 kDa) is much lower than that of other archaeal α-glucosidases. A thermophilic bacterium, *Bacillus caldovelox*, has an α-glucosidase which has a molecular mass of 30 kDa [24]. This value is similar to that of *T. hydrothermalis* α-glucosidase.

Using Blast2 (EMBL) to search databases, no similarity scores was found between *T. hydrothermalis* α-glucosidase and any α-glucosidase or polysaccharidases. Only one protein having a significant similarity score was identified. A *P. horikoshii* hypothetical protein (PH1373) (probability 2.2×10^{-48}) showed 64% similarity and 42% identity to *T. hydrothermalis* α-glucosidase through the entire sequence (Fig. 3). Only two gaps were introduced in the alignment. In his PhD thesis, Legin isolated and characterised an intracellular α-glucosidase from *T. hydrothermalis* [12]. We named our α-glucosidase 'enzyme II' to distinguish it from the intracellular 'enzyme I'. There are several differences between the two enzymes in terms of size (23.7 against 110 kDa) and N-terminal sequence. The enzyme I N-terminal sequence is AELNFKAIEEKWQKRWLEEKAFEPK [12], which is different from that of enzyme II. Several forms of α-glucosidases have been described in plants or eubacteria but it is the first time that this has been shown in archaea. Identically, it has been shown that *P. furiosus* has two α-amylases which are different in terms of size, cellular localisation and primary structure [25,26].

The cloned gene was also used as a genetic probe to determine the presence of homologous sequences in other archaeal species. Several genomic DNA from Thermococcales (*T. celer* and *T. fumicolans*) and Pyrococcales (*P. furiosus, P. abyssi* 549 and *P. abyssi* 855) strains were digested by *Hin*dIII. Except for *P. furiosus*, we found hybridisation with all these genomic DNAs. According to these results, it seems that a homologous sequence of α-glucosidase gene exists in *T. celer, T. fumicolans, P. abyssi* 549 and *P. abyssi* 855. So, as has already been shown by sequence comparison with an ORF from *P. horikoshii*, several other archaeal species possess a similar nucleotide sequence.

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