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Short communication

First crenarchaeal chitinase found in *Sulfolobus tokodaii*

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ABSTRACT

This is the first description of a functional chitinase gene within the crenarchaeotes. Here we report of the heterologues expression of the ORF BAB65950 from *Sulfolobus tokodaii* in *E. coli*. The resulting protein degraded chitin and was hence classified as chitinase (EC 3.2.4.14). The protein characterization revealed a specific activity of 75 mU/mg using colloidal chitin as substrate. The optimal activity of the enzyme was measured at pH 2.5 and 70 °C, respectively. A dimeric enzyme configuration is proposed. According to amino acid sequence similarities chitinases are attributed to the two glycoside hydrolase families 18 and 19. The derived amino acid sequence of the *S. tokodaii* gene differed from sequences of these two glycoside hydrolase families. However, within a phylogenetic tree of protein sequences, the crenarchaeal sequence of *S. tokodaii* clustered in close proximity to members of the glycoside hydrolase family 18.

1. Introduction

Chitin is after cellulose the second most abundant biopolymer on earth, consisting of beta-1–4-glycosidic bonded N-acetyl-glucosamine subunits with various grades of acetylation. It is wide spread from deserts to the deep sea, generated mostly by arthropoda and fungi with a production and steady state amount of an estimated 10^{10} to 10^{11} tons per year (Patil et al. 2000). Chitin degradation is an extremely important step in nutrient cycling especially in the oceans (Poulicek and Jeuniaux 1991) and comprises the combined action of several enzymes. During the degradation process chitinases (EC 3.2.4.14) mainly hydrolyse the beta-1,4-glycosidic bonds within the chitin polymer and are attributed to the glycoside hydrolase (GH) families 18 and 19 (Henrissat 1991) according to sequence homologies.

Family 18 harbours mainly chitinases from bacteria and is further divided into the three subfamilies A, B and C (Karlsson and Stenlid 2009). These chitinases consist in general of one catalytic domain and one or more chitin binding domains. The enzyme family is characterized by its barrel structure (TIM Barrel) consisting of eight alpha-helices and eight parallel beta-strands that alternate along the peptide backbone. The catalytic residue has been identified experimentally to be a glutamic acid (Tsuji et al. 2010).

Family 19 comprises mainly plant chitinases. The members of this enzyme family have a bilobal structure with a high content of alpha-helices (Prakash et al. 2010). The catalytic residue of the enzyme class has not been verified yet.

Although chitinases are widely distributed in all domains of life, only little is known about archaeal chitinases. Within the domain of archaea, only ten euryarchaeal chitinases were found so far in terms of genetic or molecular information (Table 1). All were attributed to glycoside hydrolase subfamilies 18A and 18C. Most of them were annotated by genome mining using similarity to known genes. Thus, their actual chitin degrading capabilities have mostly not been elucidated. To our knowledge, enzymatic characterization including proof of activity has been accomplished for four organisms only:

(1) In *Thermococcus chitinophagus*, the only archaeon shown to grow directly on chitin as carbon and energy source so far, a chitinase bound to the outer side of the cell was detected. Its optimal activity was measured at 70 °C and pH 7. The chitinase was highly thermostable and showed no inhibition by allosamidin. In addition, it was resistant to denaturation by urea and SDS (Andronopoulou and Vorgias 2004).
(2) Tanaka et al. (2001) cloned and overexpressed the chitinase gene of *Thermococcus kodakaraensis* KOD1. The enzyme showed optimal activity at 85 °C and pH 5. It had dual active sites, with one site showing similarities to GH18 A and the other one showing similarities to GH18 C and three substrate binding sites, according to the amino acid sequence and gene deletion experiments (Tanaka et al. 2001).
(3) The genome of *Pyrococcus furiosus* supposedly comprised two chitinases, belonging to the subfamilies GH18 A and B. Both were cloned and expressed in *E. coli*. They showed pH optima at pH 6 with thermal optima between 90 °C and 95 °C. Furthermore, these two chitinases acted synergistically, when incubated together on colloidal chitin, resulting in a fivefold increase compared to incubation with only one of the two

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Table 1
List of archaeal chitinases described until 2011.

Organism	Enzyme family	Type of characterization	Reference
Thermococcus chitinophagus	GH18	Native purification, activity confirmed, characterized.	GenBank Acc. No. AAR13021.1
Thermococcus kodakaraensis KOD1	GH18 A/GH18 C	Recombinant enzyme, activity confirmed, characterized.	GenBank Acc. No. BAD85954.1
Pyrococcus furiosus	GH18 A	Recombinant enzyme, activity confirmed, characterized.	GenBank Acc. No. AAL81357.1
Halobacterium sp. strain NRC-1	GH18 A	Recombinant enzyme, activity confirmed.	GenBank Acc. No. AAG19274.1
Halobacterium salinarum	GH18 A	Genome annotation.	GenBank Acc. No. CAP13543.1
Halomicrobium mukohataei DSM 12286	GH18 A	Genome annotation.	GenBank Acc. No. ACV49028.1
Haloterrigena turkmenica DSM 5511	GH18	Genome annotation.	GenBank Acc. No. ADB61056.1
Methanoplanus petrolearius DSM 11571	GH18 A	Genome annotation.	GenBank Acc. No. ADN37298.1
Candidatus Korarchaeum cryptofilum OPF8	GH18	Genome annotation.	GenBank Acc. No. ACB07477.1
Candidatus Methanoregula boonei 6A8	GH18	Genome annotation.	GenBank Acc. No. ABS56694.1

proteins (Gao et al. 2003). However, the subsequent DNA sequence analysis showed that the two genes were formed as a result of a nucleotide insertion, causing a frame shift. After removal of the inserted nucleotide, an artificial recombinant chitinase was expressed by Oku and Ishikawa (2006), resulting in a 40 fold increase in chitinase activity.

(4) Hatori et al. (2006) found a putative chitinase gene in the genome of *Halobacterium* sp. strain NRC-1 and expressed it in the extremely halophilic archaeon *Haloarcula japonica* strain TR-1. The enzyme was reported to be halophilic, with an optimal activity at about 1 M NaCl. The activity was retained at salt concentrations ranging up to approximately 5 M NaCl. In addition the enzyme was insensitive to DMSO concentrations of up to 30% (v/v).

Additionally, six sequences of putative chitinases were annotated, all from euryarchaeal organisms. Until now, no chitinases or the respective genes were described or annotated within the crenarchaeal group.

Here we show that the ORF BAB65950 from *Sulfolobus tokodaii* str. 7 encodes for the first functional crenarchaeal chitinase. We present first results concerning the enzyme properties and show that the derived amino acid sequence of the enzyme could neither be attributed to the glycoside hydrolase family 18 nor 19. However, within a phylogenetic protein sequence tree, the deduced amino acid sequence of the ORF clustered into close proximity of members of the glycoside hydrolase family 18.

2. Materials and methods

2.1. Identification of the S. tokodaii chitinase gene

Using BlastP, the amino acid sequence of the exochitinase from *Streptomyces olivaceoviridis* was compared with available archaeal sequences in Genbank, the National Centre for Biotechnology Information (NCBI) online database. One chitinase gene was identified in the completely sequenced genome of *S. tokodaii*, the ORF BAB65950, predicted to encode for a 709 amino acid long hypothetical protein.

2.2. Sequence handling

The physical and chemical properties of the *S. tokodaii* chitinase gene product were predicted using the ProtParam tool on the ExPASy Proteomics Server (Gasteiger et al. 2003). To further clarify the emerging secondary structures, the amino acid sequence was submitted to the PSIPRED Protein Structure Prediction Server (Bryson et al. 2005) and analysed using the PSIPRED v3.0 program (McGuffin et al. 2000). Amino acid sequences of families 18 and 19 with the respective subfamilies were obtained from the Carbohydrate Active Enzymes Online Database (CAZy, Cantarel et al. 2009). In addition, conserved domains of the glycoside hydrolase families 18 and 19 were obtained from NCBI Genbank. Sequence alignments were constructed with the Neighbour-joining method of ClustalX (Thompson et al. 1997) using the GONNET matrix. Phylogenetic trees were constructed using both the Neighbour-joining option of ClustalX as well as the Maximum-likelihood method of PROML (Phylip, version 3.6). Confidence limits were estimated by 1000 bootstrapping replicates.

2.3. DNA extraction

S. tokodaii strain DSMZ 16993 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) as vacuum dried culture. The culture was dissolved in 50 μl DNA free water. Total DNA was extracted with the innuPREP Bacteria DNA Kit (Analytik Jena). Crude DNA extract was purified by using the QIAamp DNA Mini Kit (QIAGEN), as described by the manufacturer.

2.4. PCR

PCR primers flanking the start and stop codon of the putative chitinase genes were designed according to the nucleotide sequence of *S. tokodaii* str. 7, obtained from the NCBI home page. Primer sequences were: SkChi f: 5′-ATG AAA CGG AAT ACC CTT TTG-3′ and SkChi r: 5′-TTA CCA ATA GTT ATC ACT TCT TTC TC-3′. For amplification of the sequences, Phusion High-Fidelity DNA polymerase (Fynnzyme) was used with concentration of 1 U per reaction and Phusion HF buffer. All primers were used in a 10 μM concentration. Thermal protocols were conducted in a T1 thermocycler (Whatman Biometra).

Cycler conditions were as follows: 1 cycle: Initial Denaturation, 98 °C, 30 s. 25 cycles: Denaturation, 98 °C, 10 s; Annealing, 50 °C, 30 s; Elongation, 72 °C, 90 s. 1 cycle: Final Elongation, 72 °C, 600 s.

2.5. Colony screening

Clones were screened for positive insert with the primers pQE30 f (5′-GAA-TTC-ATT-AAA-GAG-GAG-AAA-3′), pQE30 r (5′-ATC-CAG-ATG-GAG-TTC-TGA-GG-3′). Cycler conditions were as follows: 1 cycle: Initial Denaturation, 94 °C, 120 s. 30 cycles: Denaturation, 94 °C, 40 s; Annealing, 50 °C, 40 s; Elongation, 72 °C, 60 s. 1 cycle: Final Elongation, 72 °C, 300 s.

2.6. DNA fragment size, concentration and purity

The length of the respective DNA fragments was checked on a 2% agarose gel with a 1 kb ladder (Fermentas) as size marker. Gels were stained with SYBR safe DNA gel stain (Invitrogen) and evaluated on a UV transilluminator at 366 nm. DNA concentration and purity were determined photometrically with a NanoVue spectrophotometer (GE Healthcare).

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2.7. Cloning of archaeal chitinases

The PCR products were purified using the MinElute PCR Purification Kit (QIAGEN). The *S. tokodaii* DNA was prepared for cloning by adding "A" overhangs with the QIAGEN A-Addition Kit (QIAGEN). The PCR product was ligated into the vector pQE-30UA (QIAGEN) with the QIAGEN UA Cloning Kit (QIAGEN) and transformed into chemically competent *E. coli* JM109 cells (Stratagen). Cells were grown on LB medium (Miller 1972) containing 50 μg/ml carbenicillin. Colonies were picked and screened for the correct insert by using the described colony PCR method.

2.8. Overexpression of recombinant chitinases

The *S. tokodaii* plasmid was purified and transferred into the expression strain *E. coli* BL21 cd+ (Stratagen). The clones were grown in liquid LB medium (Miller 1972) containing 50 μg/ml carbenicillin and 100 μg/ml chloramphenicol at 37 °C, 120 rpm. Overexpression was induced by addition of 0.4 mM IPTG as the cell culture reached an optical density of 1. Cells were harvested by centrifugation after 18 h of further growth.

2.9. Purification of the recombinant archaeal chitinases

The harvested *E. coli* cells were resuspended in 0.1 M citric acid, pH 3. The cells were ruptured with a French pressure cell at 18,000 PSI (SLM Aminco, G. Heinemann). After cell lysis, the sample was centrifuged at 17,700 × *g* for 30 min (Beckman). The supernatant was incubated at 60 °C for 1 h, followed by a second centrifugation step at 17,700 × *g* for 30 min. The supernatant was diluted in 0.1 M Tris/HCl buffer, pH 8.5 and applied to a HiLoad 26/10 Q Sepharose High Performance ion exchange column (GE Healthcare), equilibrated in 0.1 M Tris/HCl buffer, pH 8.5. The respective protein was eluted by a gradient of 0–2 M NaCl in 0.1 M NaAc buffer pH 5, with a flow rate of 8 ml/min. Active fractions were concentrated on a 30 kDa filter (Millipore). The obtained concentrate was applied to a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare), equilibrated in 0.1 M NaAc buffer pH 5 containing 150 mM NaCl. Elution was performed with a flow rate of 1 ml/min to obtain the fraction containing the purified enzyme. Fractions were tested for chitinase activity and purity of enzyme as described below.

2.10. Characterization of the purified S. tokodaii chitinase

The enzyme activity was tested using the 3-methyl-2-benzothiazoline hydrazine (MBTH) assay (Horn and Eijsink 2004). The conversion of chitin to the respective oligomers was measured with N-acetyl-glucosamine as standard. One unit was defined as the release of 1 μmol GlcNAc per minute. The pH optimum was determined by the incubation of the *S. tokodaii* enzyme (0.014 mg/ml) with buffers reaching from pH 3 to pH 7 (0.1 M sodium citrate for pH 3 and pH 4; 0.1 M NaAc for pH 5–7) in discrete steps of 1 pH at 28 °C. The temperature optimum was determined by incubation of the protein (0.017 mg/ml) at temperatures ranging from 30 to 80 °C in discrete steps of 10 °C at pH 5 (0.1 M NaAc buffer). K_M and v_{max} values were determined by incubation of the enzyme (0.026 mg/ml) in 0.1 M NaAc buffer pH 5 at 28 °C with concentrations of colloidal chitin from 0.5 to 5 mg/100 μl in discrete steps of 0.5 mg. The results were used with a Lineweaver–Burk linearisation to calculate K_M and v_{max} values.

2.11. Size calculation of the S. tokodaii chitinase

The size of the native protein was calculated according to the retention time on a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare). The column was calibrated with dextran blue (2000 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa) (all from GE HEalthcare), amylase (200 kDa), and bovine serum albumin (fraction V, 66 kDa).

2.12. SDS PAGE

The enzyme purity at various steps of the purification procedure and the molecular mass of the single subunit of the protein were checked by SDS-PAGE in 12% polyacrylamide gels followed by staining with Coomassie brilliant blue R 250 according to standard procedures (Lämmli 1970).

2.13. Protein concentration

Protein concentration was determined with the Coomassie dye binding assay according to Zor and Selinger (1996) with bovine serum albumin fraction V (Sigma) as standard.

3. Results

3.1. Identification of the chi gene from S. tokodaii

Based on amino acid sequence similarity, an open reading frame (ORF) was identified by a BLAST P search in the completely sequenced genome of *S. tokodaii*. This ORF, BAB65950, was predicted to encode for a "709 amino acid long hypothetical protein". The ORF contained 2130 bp coding for a polypeptide of 709 amino acids with a calculated molecular mass of 77.7 kDa. The protein had a predicted pI of 8.32. The G + C content of the ORF was 33%. The coding sequence started with ATG and stopped with TAA.

3.2. Sequence analysis of S. tokodaii

The primary structure of the gene was subjected to a BLAST search, upon which the most similar enzymes as well as the most similar three-dimensional (3D)-structure-determined enzymes were identified. The domain organisation and presence of conserved motifs were analysed and compared using a combined alignment (Fig. 1). The *S. tokodaii* sequence could be neither matched with the highly conserved DXDXE signature motif of the GH18 family (Tsuji et al. 2010), nor with the highly conserved [FHY]-G-R-G-[AP]-X-Q-[IL]-[ST]-[FHYW]-[HN]-[FY]-NY motif of the GH19 family (Huet et al. 2008). As this is the first crenarchaeal chitinase, the sequence of the *P. furiosus* chitinase was added to the alignment for comparison of possible similarities. However no highly conserved regions were found that are shared between the two archaeal protein sequences. At the N-terminal end of the *S. tokodaii* protein, a broad-complex, tramtrack and bric a brac (BTB) domain (Bardwell and Treisman 1994) was found. Adjacent to the BTB domain, a chitin/cellulose binding domain (ChtBD3) (Brun et al. 1997) with the conserved residues Trp220 and Tyr237 was identified. In the middle of the *chi* sequence, a fibronectin type III domain was detected (Toratani et al. 2006).

An additional alignment of the *S. tokodaii* protein sequence with the available sequences of *Sulfolobus acidocaldarius* (ORF: AAY80633.1), *Sulfolobus islandicus* (ORF: YP_003419346.1) and *S. solfataricus* (ORF: YP_002840670.1) showed only similarities in the fibronectin type III region (data not shown).

3.3. Glycoside hydrolase affiliation of the crenarchaeal chitinase

In total, 70 sequences from GH family 18 ($n = 50$) and 19 ($n = 20$) were obtained from NCBI (Table S1, supplementary material) and aligned with ClustalX. The sequences of the GH18 and GH19 family clustered apart and formed distinct group. Within the GH18 family

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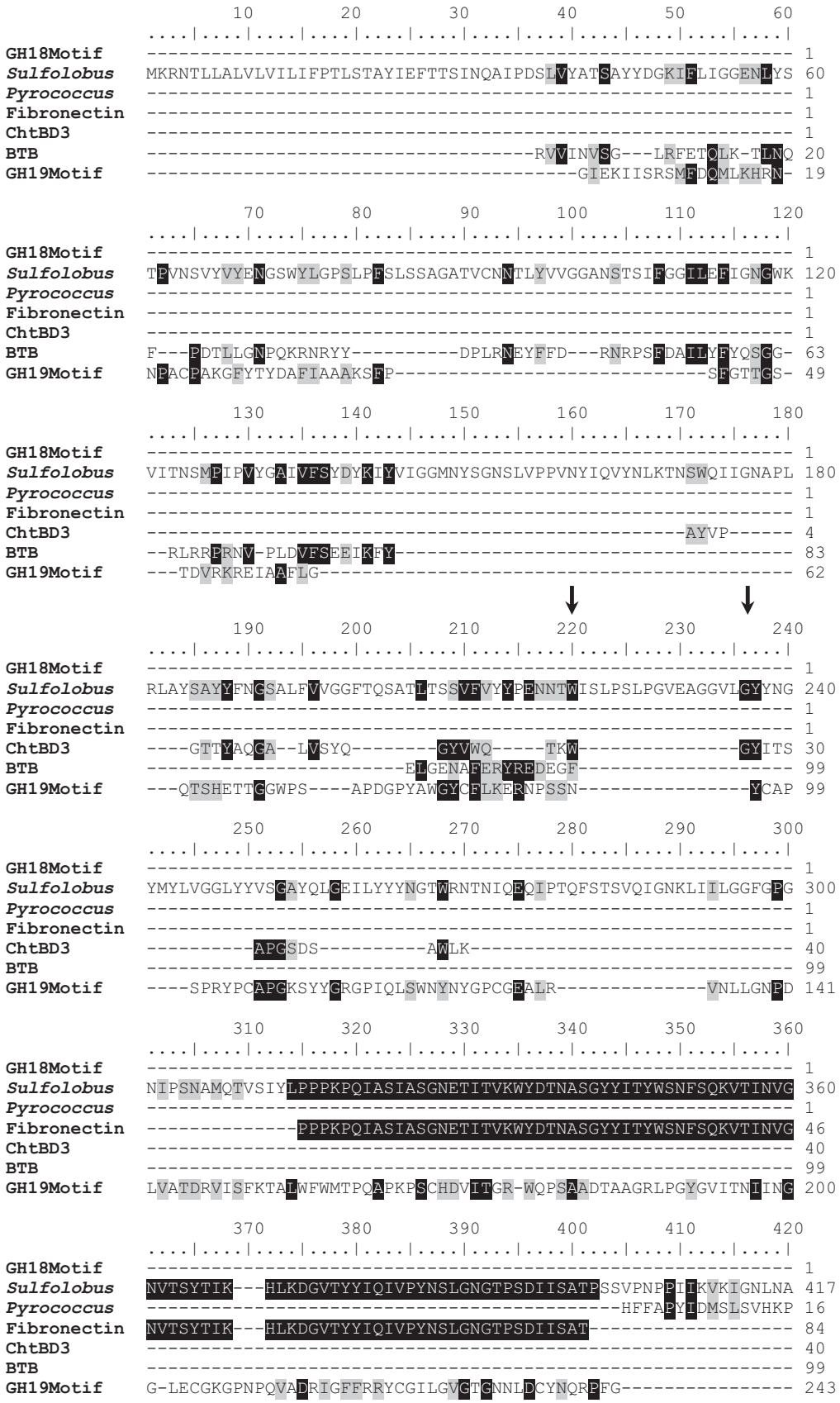

Fig. 1. Protein sequence similarity of proposed *Sulfolobus tokodaii* chitinase with chitinase core motifs. Black boxes denote conserved amino acids; grey boxes denote similar amino acids. GH18 motif: consensus GH18 core motif with signal sequence and conserved glutamic acid residue (Tsuji et al. 2010). *Sulfolobus*: amino acid sequence of ORF BAB65950 from *Sulfolobus tokodaii* (NCBI accession number: NC 003106). *Pyrococcus*: amino acid sequence of the *Pyrococcus furiosus* chitinase (NCBI accession number: NC 003413). Fibronectin: consensus amino acid sequence of conserved fibronectin domain (Toratani et al. 2006). ChtBD3: consensus amino acid sequence of conserved

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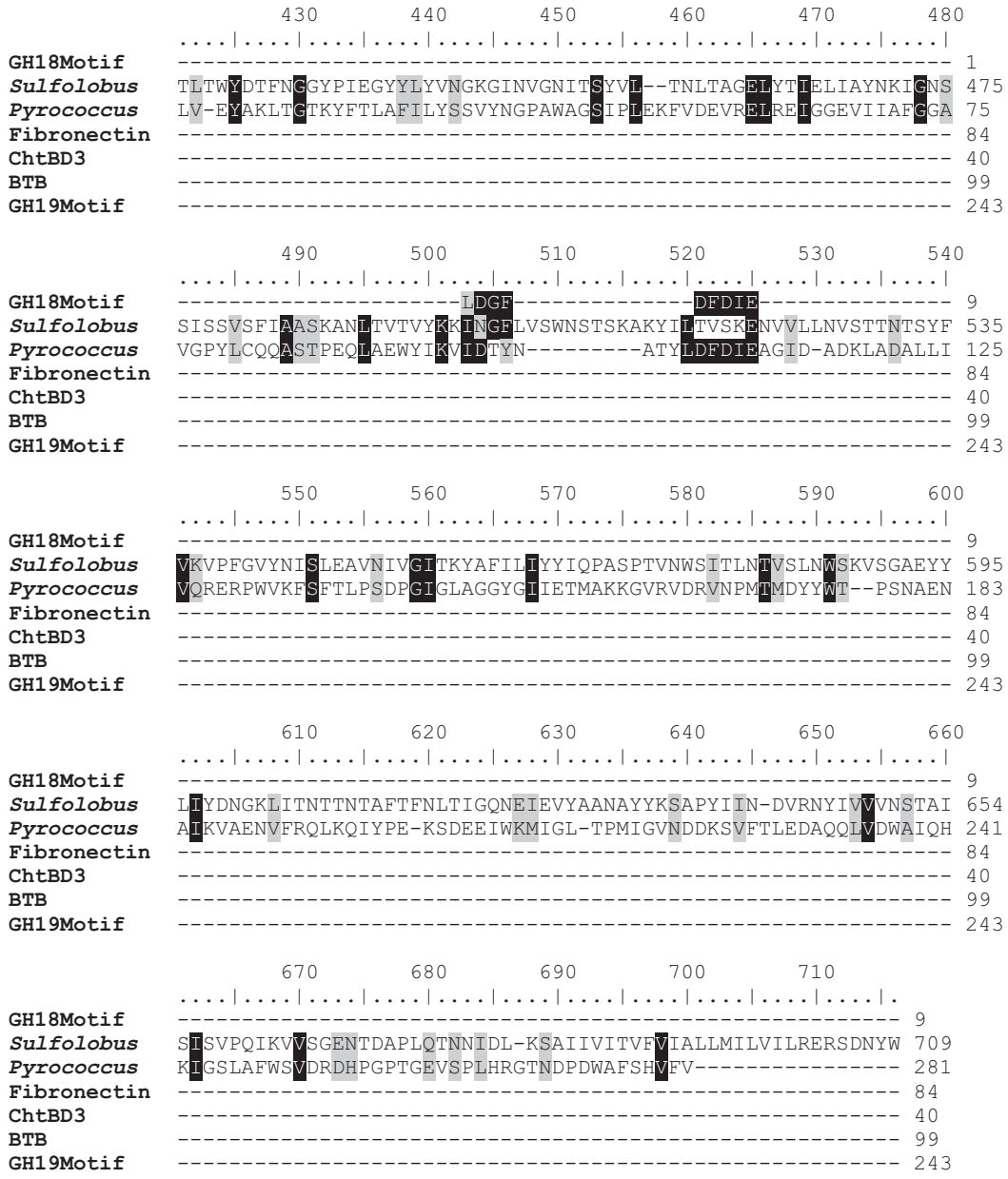

Fig. 1. (*Continued*).

the three distinct subfamilies GH18 A, B and C were detected. The euryarchaeal sequences were found within the GH18 clade in the subfamilies A and C. The crenarchaeal *S. tokodaii* chitinase clustered into close proximity to members of the GH 18 family (Fig. 2).

3.4. Cloning and overexpression of the chitinase gene from S. tokodaii

The *chi* gene was amplified by PCR and cloned into the vector pQE-30UA (QIAGEN). The recombinant plasmid was used to transform *E. coli* Bl21 cd+. Expression was induced by IPTG. The overexpressed protein was examined by SDS-PAGE, indicating a single band with a molecular mass of approximately 77 kDa. This result was consistent with the molecular mass predicted from the nucleotide sequences (77.7 kDa). The recombinant protein was purified by heat and acid precipitation, followed by a cation exchange and a size exclusion chromatography. According to the retention time of the protein, the native molecular mass was determined to be 130 kDa.

3.5. Kinetic characterization of the S. tokodaii enzyme

Utilising the MBTH method a specific activity of 75 mU/mg could be detected in the purified protein (Table 2). Its optimal activity was measured at 70 °C and pH 2.5. The chitinase K_M value was determined to be 65.9 mg of colloidal chitin (data not shown).

chitin binding domain (Brun et al. 1997) with conserved binding residues Trp220 and Tyr237 (black arrows, the numbering of these residues refers to the position within the *Sulfolobus tokodaii* protein). BTB: consensus amino acid sequence of the broad-complex, tramtrack and bric a brac domain (Bardwell and Treisman 1994). GH19 motif: consensus GH19 motif (Huet et al. 2008).

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Table 2
Measured release of N-acetyl-glucosamine, incubation time, protein content and resulting specific activity after different purification steps of the *Sulfolobus tokodaii* chitinase. Values are given rounded to the second decimal.

Purification step	Release of GlcNac (mM)	Incubation time (min)	Protein (mg/ml)	Specific activity (mU/mg)
Raw extract	0.35	1020	0.42	0.83
60 °C precipitation	0.42	1020	0.3	1.38
Q Sepharose column	0.26	751	0.03	12
Superdex 200 column	0.57	1215	0.01	75

4. Discussion

With our experiments, we were able to show that the protein encoded by the ORF BAB65950 from *S. tokodaii* degrades chitin. Hence it can be classified as the first crenarchaeal chitinase. This classification is also supported by the presence of detected amino acid motifs characteristic for chitinases. This work shows the first results of the characterization of this novel crenarchaeal protein.

4.1. The S. tokodaii chi sequence

Chitinase related sequence motives were found in the derived amino acid sequence from the ORF BAB65950. Neither the GH18 specific TIM barrel structure, nor the bilobal structure of the GH19 family with its high alpha helical content could be found in the predicted secondary structure. The affiliation of the protein to the chitobiase family (GH20) is unlikely, as the GH20 enzyme class does not act primarily on polymeric substrates (Hoell et al. 2010) and

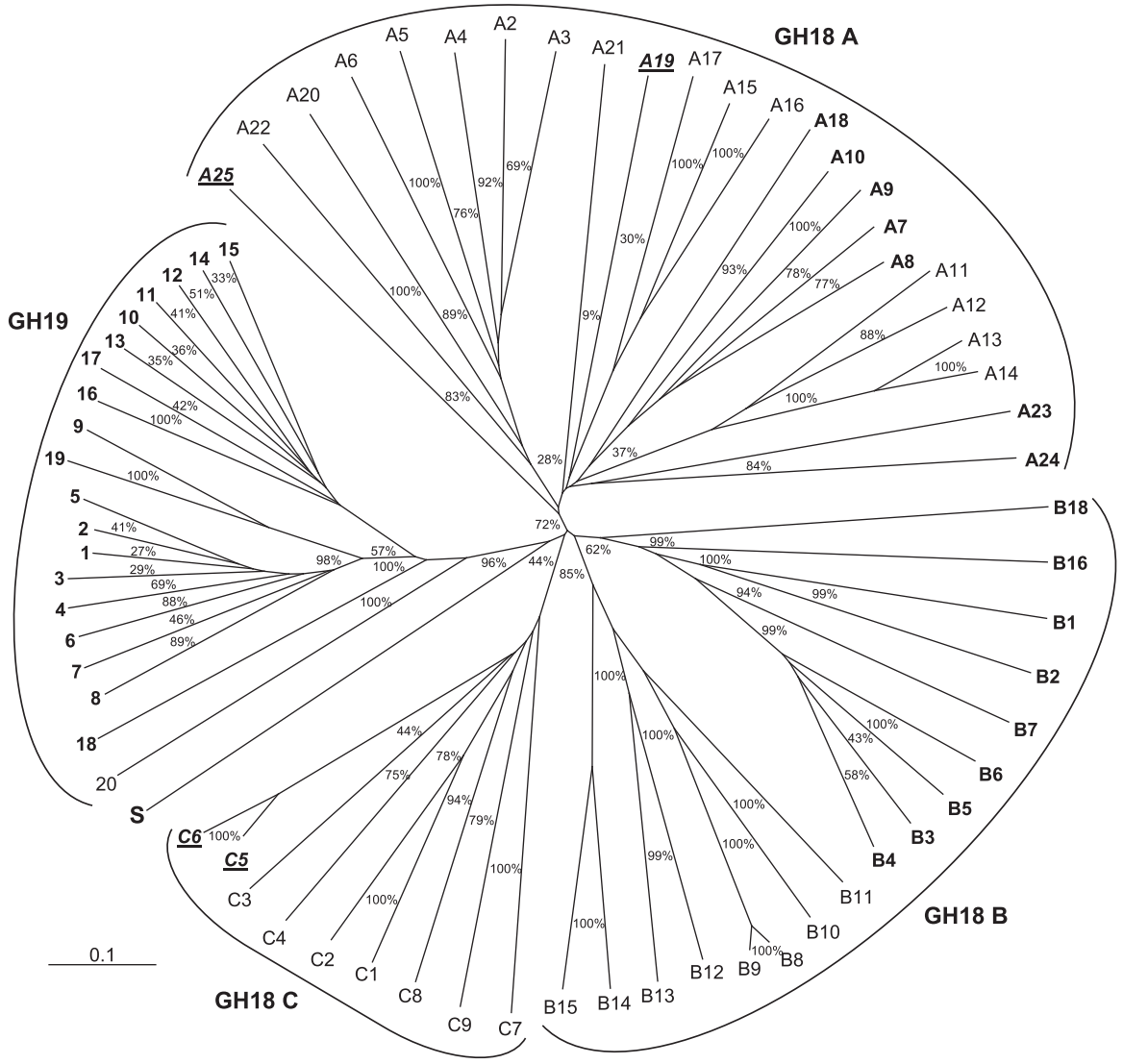

Fig. 2. Amino acid sequence similarity tree based on 70 sequences from GH family 18 ($n = 50$) and 19 ($n = 20$). The phylogenetic tree was constructed using both the Neighbour-joining option of ClustalX (Thompson et al. 1997) as well as the maximum-likelihood method of PROML (Phylip, version 3.6). Abbreviations: GH18 A: subfamily 18 A. GH18 B: subfamily 18 B. GH18 C: subfamily 18 C. GH19: GH family 19. S: *Sulfolobus tokodaii* sequence. Printed in bold: Eukaryal sequences. Printed in bold italics and underlined: *Euryarchaeal* sequences. Sequence abbreviations and respective Genbank accession numbers are given in supplementary material (Table S1). Bootstrap values (1000 replicates) are given in *per cent* between the respective branches.

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contains also the TIM barrel structure within its catalytic domain (Tews et al. 1996). The detected BTB-domain (broad-complex, tramtrack and bric a brac) is known as a protein–protein interaction motif (Bardwell and Treisman 1994) and plays a role in dimerisation. In accordance, the native molecular weight of the *S. tokodaii* protein was determined to be 130 kDa, indicating a dimeric composition of the native protein. A dimeric status is also known from other chitinases, such as the *P. furiosus* chitinase (Nakamura et al. 2007). Hence the found BTB domain is proposed as linking region. This hypothesis has to be further tested. A N-terminal deletion should decrease the native mass form 130 kDa to the expected 77 kDa and confirm our findings. The adjacent chitin/cellulose binding domain (ChtBD3) is known from many different glycoside hydrolase enzymes (Brun et al. 1997) and might be crucial for the enzymes carboactive properties. The detected highly conserved fibronectin type III domain is typical for bacterial chitinases and reported to be also involved in substrate binding (Toratani et al. 2006). Also the question remains, whether the enzyme is excreted or not, as no leader sequences were detected.

4.2. Glycoside hydrolase family affiliation

The difference of the *S. tokodaii* chitinase to the glycoside hydrolase families 18 and 19 harbouring the known chitinases was supported by the phylogenetic analysis (Fig. 2). Both glycoside hydrolase families show completely different structures and are not believed to be related. The crenarchaeal sequence of *S. tokodaii* did neither cluster clearly with the GH18 nor the GH19 family. The enzyme has to be further analysed to clarify its glycoside hydrolase family affiliation.

Concerning the affiliation of all archaeal chitinase sequences, the archaeal chitinases formed no domain specific group as described for other enzymes of sugar metabolism, such as glucokinases or pyruvate kinases (Siebers and Schönheit 2005), but cluster with functional but not phylogenetic groups.

As the detected conserved domains within the amino acid sequence are not exclusively found in chitinases and as the enzyme could not be clearly assigned to one of the chitin containing glycoside hydrolase families based on sequence similarities, a molecular based search alone would not suffice to postulate a chitinolytic activity of this enzyme in our opinion. Hence the enzyme was also expressed to verify a potential chitinolytic activity.

4.3. Comparison of the found crenarchaeal chitinase with other archaeal chitinases

Within the domain of archaea, only ten euryarchaeal chitinases were found so far in terms of genetic or molecular information. Most of them were annotated by genome mining in analogy to known genes. Thus, their actual chitin degrading capabilities have not been elucidated yet. To our knowledge, enzymatic characterization including proof of activity has been accomplished only for the chitinases from *P. furiosus*, *T. kodakaraensis* KOD1, *T. chitinophagus* and *Halobacterium salinarum* strain NRC-1. The activity of the *S. tokodaii* protein (75 mU/mg) was in the same range as the activities of the reported euryarchaeal chitinases from *P. furiosus* and *T. kodakaraensis* KOD1. The *P. furiosus* chitinases ChiA and ChiB showed a specific activity of 35 mU/mg (Gao et al. 2003), whereas the *T. kodakaraensis* KOD1 chitinase had a specific activity of 18 mU/mg (Tanaka et al. 2001). The chitinolytic activity of the detected *S. tokodaii* chitinase was forty-fold lower as compared to the natively purified chitinase from *T. chitinophagus* with a specific activity of 3 U/mg. This chitinase is the only non-recombinant archaeal chitinase described so far (Andronopoulou and Vorgias 2004). The difference of the detected chitinolytic activity may be inherent to the technique of recombinant protein production and might be overcome by using the archaeal expression systems with *Sulfolobus solfataricus* as host instead of *E. coli*. In addition, a chitinase was found in the genome of *H. salinarum* strain NRC-1 (Hatori et al. 2006). The enzyme was reported to be halophilic, with an optimal activity at about 1 M NaCl. The activity was retained at salt concentrations ranging up to approximately 5 M NaCl. The enzyme was insensitive to DMSO concentrations of up to 30% (v/v). However a specific activity was not given and hence could not be compared to our results.

Furthermore the *S. tokodaii* chitinase showed optimal activity at 70 °C and pH 2.5 and was classified as thermoacidophiolic. This enzyme optimum corresponds with the natural living conditions of *S. tokodaii* dwelling in sulphur rich hot acid springs in volcanic regions. Its optimal growth conditions are aerobic, at 80 °C with a low pH (2–3). This might be a hint for the expression of the enzyme by *S. tokodaii* in its natural environment. However, this hypothesis needs still to be tested by cultivation experiments.

5. Conclusions

Within the genome of *S. tokodaii* we found the first functional crenarchaeal chitinase. These findings were supported by the detected release of GlcNAc from chitin by the enzyme and the presence of several chitinase characteristic amino acid sequence motifs. Based on these first results the chitinase can be classified as thermoacidophillic. Many questions concerning the enzyme properties still remain and have to be answered in further studies.

The derived amino acid sequence of the enzyme could not be attributed to the two major chitinase containing glycoside hydrolase families 18 or 19. Hence further studies have to be undertaken to elucidate the phylogenetic position of this crenarchaeal protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.micres.2011.11.001.

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