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Cloning, Sequencing, Characterization, and Expression of an Extracellular α-Amylase from the Hyperthermophilic Archaeon *Pyrococcus furiosus* in *Escherichia coli* and *Bacillus subtilis**

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A gene encoding a highly thermostable extracellular α-amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* was identified. The gene was cloned, sequenced, and expressed in *Escherichia coli* and *Bacillus subtilis*. The gene is 1383 base pairs long and encodes a protein of 461 amino acids. The open reading frame of the gene was verified by microsequencing of the recombinant purified enzyme. The deduced amino acid sequence is 25 amino acids longer at the N terminus than that determined by sequencing of the purified protein, suggesting that a leader sequence is removed during transport of the enzyme across the membrane. The recombinant α-amylase was biochemically characterized and shows an activity optimum at pH 4.5, whereas the optimun temperature for enzymatic activity is close to 100 °C. α-Amylase shows sequence homology to the other known α-amylases and belongs to family 13 of glycosyl hydrolases. This extracellular α-amylase is not homologous to the subcellular α-amylase previously isolated from the same organism.

One of the most abundantly distributed polysaccharides in nature is starch, which is produced by plants; it is composed of two high molecular weight compounds, amylose and amylopectin. Amylose is a linear chain of glucose residues linked with an α-1,4 bond. Amylopectin is a branched polymer where the α-1,4-linked glucose residues are branched every 17–26 residues with α-1,6-linked points.

A wide variety of microorganisms are able to degrade and utilize this natural high molecular weight biopolymer by secreting starch-degrading enzymes. These enzymes act either from the nonreducing end of the chain acting as exo-enzymes producing low molecular weight products (*i.e.* β-amylase, glucoamylase, and α-glucosidase) or in the interior of the chain and in a random fashion acting as endo-enzymes and producing linear and branched saccharides with various lengths (*i.e.* α-amylase).

A great number of α-amylases (E.C. 3.2.1.1) have been isolated from a variety of eucaryotic and procaryotic organisms, and they are described in a previous reports (1, 2). All of them have been compiled in family 13 of the classification of the glycosyl hydrolase superfamily described in Ref. 3. Two α-amylases from *Dictyolglomus thermophilum* (4) and *Pyrococcus furiosus* (5, 6), could not be classified in family 13 and have been included in newly established family 57.

P. furiosus is an anaerobic marine heterotroph growing optimally at 100 °C and initially was isolated and characterized (7). Two reports on the identification of α-amylase activity from cell homogenates as well as in the culture medium have been published (8, 9) from this organism. One α-amylase, which is a subcellular enzyme, has been purified (5), cloned, and overexpressed in *Escherichia coli* (6).

In this paper, we present the gene isolation, gene cloning, sequencing, and expression in *E. coli* and *Bacillus subtilis* and biochemical characterization of a new extracellular α-amylase from *P. furiosus*. Primary structure analysis and comparison with known α-amylase revealed that this enzyme belongs to family 13 of glycosyl hydrolases and does not show sequence homology to its subcellular counterpart from the same organism.

MATERIALS AND METHODS

Chemicals and Media—Chemical for gel electrophoresis were from Serva (Heidelberg, Germany); the various substrates were from Fluka (Buchs, Switzerland). Restriction endonucleases were purchased from New England Biolabs and Boehringer Mannheim and used as recommended by the manufacturers. T4 DNA ligase was purchased from New England Biolabs and used as recommended by the manufacturer. All the other chemicals were from Merck (Darmstadt, Germany).

The medium for visualization of amylase activity was Luria Broth agar containing (per 500 ml of agar) 10 ml of a dyed amylopectin solution prepared as follows: 12.5 g of amylopectin (Serva 2000–4000 kDa) was dissolved by boiling in 250 ml of water and cooled to room temperature, 30 ml of 4 M NaOH and 2.5 g of Cibacron Rot B were added, and the solution was incubated overnight. pH was adjusted to 7 with 4 M HCl. 500 ml of 96% ethanol was added with stirring to precipitate the amylopectin as a red, viscous precipitate. The supernatant was discarded, and the amylopectin was dissolved in 200 ml of water by slight heating. The precipitation with ethanol was repeated once more. The amylopectin was again dissolved in 200 ml of water and autoclaved and was then ready for use.

Bacterial Strains—E. coli has been described in Ref. 10, and cells were prepared for and transformed by electroporation using a Gene Pulser™ electroporator from Bio-Rad as described by the supplier. *B. subtilis* DN1885 has been described by Diderichsen *et al.* (10), and competent cells were prepared and transformed as described in Ref. 11.

Plasmids—pSJ1678 was used as cloning vector in the construction of the gene library, and pUC19 (12) was used for subclonings. The experimental techniques used to construct the plasmids were standard techniques within the field of recombinant DNA technology (13). Preparation of plasmid DNA from all strains was constructed by the method described in Ref. 14.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U96622.

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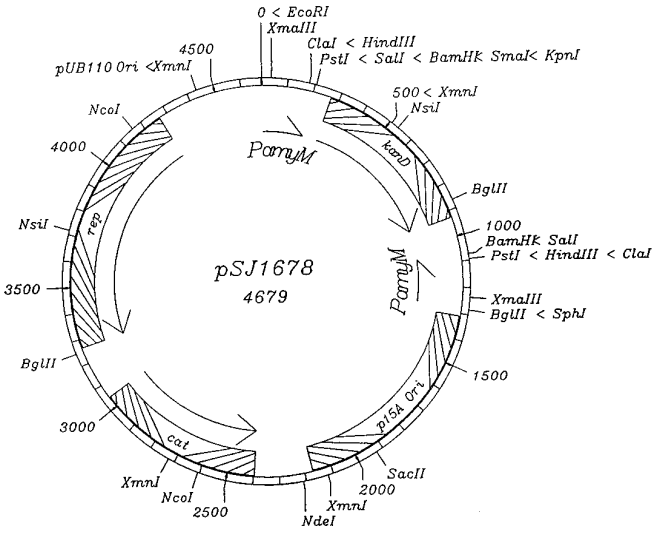

FIG. 1. **The cloning vector used was pSJ1678.** This plasmid is a shuttle vector that replicates in *E. coli* and in *Bacillus* sp. Upon cloning, the *kanD* gene fragment is removed by digestion with *Bam*HI, and the fragments to be cloned are inserted in its place. *PamyM* is the promoter from a *Bacillus* amylase, which then reads into the cloned insert from either direction to ensure the expression of the cloned genes. If nothing is cloned, the two *PamyM* promoters create an inverted repeat, and plasmids with inverted repeats of this size are not viable. There is therefore a positive selection for recombinant plasmids.

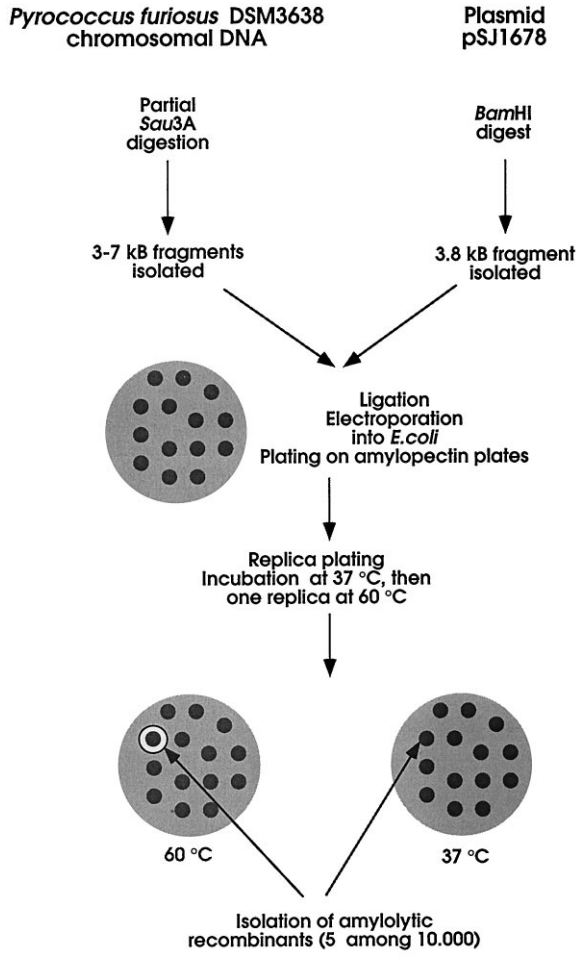

FIG. 2. **Outline of the methodology applied to isolate, clone, and detect DNA fragments of chromosomal DNA from *P. furiosus* exhibiting amylolytic activity.** A short description of this experimental approach is under "Material and Methods."

Cloning of the P. furiosus α-Amylase Gene—Genomic DNA from *P. furiosus* DSM3638 was isolated by the method described in Ref. 15. Approximately 100 μg of DNA was partially digested with *Sau*3A and size-fractionated on a sucrose gradient, and fragments between 3 and 7 kb[1] were pooled.

The cloning vector pSJ1678 (see Fig. 1) was digested with *Bam*HI, and a 3.8-kb fragment was purified from an agarose gel. Approximately 0.75 μg of vector fragment was ligated to ~4 μg of size-fractionated *P. furiosus* chromosomal DNA and used to transform *E. coli* SJ2 by electroporation.

The gene bank was plated on LB plates containing dyed amylopectin and supplemented with 6 μg/ml chloramphenicol. Following overnight incubation at 37 °C, each plate was replica plated onto two new plates, which were then incubated overnight at 37 °C. One of these was subsequently incubated at 60 °C overnight (see Fig. 2).

Subcloning of P. furiosus α-Amylase Gene—pS2467 was digested with *Cla*I, the 4.5-kb fragment containing the α-amylase gene was ligated to *Acc*I-digested pUC19 DNA, and the ligation mixture was transformed into *E. coli* SJ2. Transformants were obtained containing the insert in each of the two possible orientations with respect to the cloning vectors. These clones were SJ2481 containing the pSJ2481 construct and clones SJ2482 containing pSJ2482 construct.

Southern Analysis—pSJ2481 was ^{32}P-labeled by nick translation using a commercial kit obtained from Amersham Corp. and used as a probe in a Southern analysis. Hybridization was overnight at 60 °C in 10 × Denhardt's solution, 1% SDS, 10 mM EDTA and 5 × SSC followed by two 15-min washes in 2 × SSC, 0.1% SDS at room temperature and one 15-min wash at 60 °C (see Fig. 3).

DNA Sequencing—4.5 kb of the *P. furiosus* DNA insert clones on pSJ2467 was sequenced on both strands, using Sequenase™ (16) and a combination of subclones and oligonucleotide primers based on previously determined sequences.

Expression of the α-Amylase Gene in B. subtilis—The plasmid pSJ1678 used for construction of the gene library is a shuttle vector able to replicate both in *E. coli* and *B. subtilis*. To test for expression of the amylase activity in *B. subtilis*, pSJ2467 was therefore transformed into competent cells of DN1885 selecting for resistance to chloramphenicol (6 μg/ml) on LB plates containing dyed amylopectin. 10 transformants were picked onto two new plates with dyed amylopectin along with SJ1678, which is DN1885/pSJ1678 as a control. After incubation overnight at 37 °C, one plate was transferred to 65 °C, whereas the other was kept at 37 °C. Seven hours later, the degradation of the amylopectin around the 10 transformants with pSJ2467 was apparent on the plate incubated at 65 °C because of the formation of a clear halo. No degradation halo was formed around the control strain (see Fig. 6).

Analytical Methods—Preparative polyacrylamide gel electrophoresis was performed in 1.5-mm-thick polyacrylamide gels (either homogeneous (5 or 12%, w/v) or gradient gels (5–30%, w/v)) at a constant voltage of 400 V for 24 h at 4 °C. The protein bands were visualized by silver staining (17). Analytical 11.5% polyacrylamide slab electrophoresis in the presence 0.1% SDS was carried out at a constant current of 40 mA/gel for 3 h. Commercially available molecular weight markers were used to calibrate the gel. The protein band exhibiting α-amylase activity on the gel was detected by soaking the gels in 50 mM acetate buffer, pH 5.5, containing 1% (w/v) starch for 1 h at 4 °C, further incubating the gels at 90 °C for 30 min, and staining the gels with 0.15% (w/v) iodine and 1.5% (w/v) potassium iodide until a clear zone became visible.

Amylase activity was determined in the cell supernatant as described in Ref. 18. In a typical assay, enzyme solution up to 100 μl was added to 250 μl of sodium acetate buffer (50 mM, pH 5.5) containing 1% (w/v) starch and incubated at 95 °C for 30 and 60 min. One unit of α-amylase is defined as the amount of the enzyme that liberates 1 μmol of reducing sugar/min with maltose as a standard.

The activity- and temperature-dependent experiments were carried out in a water bath for the range between 40 and 90 °C, whereas for the range between 90 and 130 °C a glycerol bath was used. Activity tests above 100 °C were carried out in closed Hungate tubes to prevent boiling of the solution. For the determination of pH optimum activity, the following buffers were used for the different pH ranges: for pH 3.5–4.0, 50 mM citrate; for pH 4.5–6.0, 50 mM sodium acetate; and for pH 6.5–7.0, 50 mM potassium phosphate.

[1] The abbreviations used are: kb, kilobase(s); HPLC, high pressure liquid chromatography.

The substrate specificity of the α-amylase was studied in 50 mM sodium acetate at pH 5.5 using 0.5 unit of purified enzyme/ml of reaction at 90 °C for 30 min. The final concentration of various substrates was 1% (w/v). Thermal stability experiments were performed at the indicated temperatures in the above mentioned baths, and the residual activity was determined in a typical enzyme assay solution.

Sugars released by the enzymatic action of α-amylase from *P. furiosus* on starch were analyzed as follows. For each milliliter of acetate buffer (50 mM, pH 5.0), 0.5 unit of α-amylase was added. The final starch concentration was 1% (w/v), and the incubation was conducted at 90 °C. Samples were withdrawn after various time intervals. Each sample was purified with ion exchange resin (Serdolyt MB, Serva, Heidelberg, Germany), and the sugars were analyzed by HPLC using an Aminex HPX-42 A column (Bio-Rad, Richmond, CA). Sugars eluted were monitored by a differential refractometer (Knauer, Bad Homburg, Germany).

Computer Analysis—The search for sequence homology to the other amylases performed at the National Center for Biotechnology Information using the BLASTP network service (19).

RESULTS

Cultivation of P. furiosus—P. furiosus (DSM 3638) was cultivated at 98 °C in a medium as described in Ref. 20. 20-liter cultures were continuously gassed with H_2/CO_2 (80:20). Cell growth was paralleled by enzyme production and degradation of starch in the medium. By using continuous gassing, an enzyme activity above 1000 units/liter was detected. These growth conditions caused about 80% secretion of the enzyme into the culture fluid. The amount of the secreted enzyme reached the maximum after 18 h of cultivation (data not shown).

Cloning and Sequencing of the α-Amylase Gene of P. furiosus—The preparation of the DNA expression library was carried out as described under "Materials and Methods." Fig. 1 shows the pSJ1678 vector that was used to clone and express the *P. furiosus* DNA library.

Fig. 2 outlines the clone selection procedure used to isolate the α-amylase gene, which is based on the secretion of amylolytic activity from the recombinant cells and the detection of this activity around the colony(ies) by producing a clear halo. The qualitative detection of amylase activity on agar plates is also described under "Materials and Methods." Among 10,000 colonies, five colonies have shown clear halos indicating degradation of the amylopectin on the 60 °C agar plates, whereas no halos were observed around the colonies on the plates that were kept at 37 °C. These five clones were taken from the 37 °C plates and named SJ2463 through SJ2467.

Restriction digests using *Hin*dIII revealed that the *P. furiosus* DNA inserts on the four clones SJ2463, SJ2464, SJ2465, and SJ2467 shared a common DNA region without the inserts being totally identical, whereas the DNA contained on SJ2466 clone appeared unrelated to the other four clones. We used the construct pSJ2467, which contained an insert of approximately 4.5 kb, for further analysis.

pSJ2467 was digested with *Cla*I, the 4.5-kb fragment containing the α-amylase gene was ligated to *Acc*I-digested pUC19 DNA, and the ligation mixture was transformed into *E. coli* SJ2. Transformants were obtained containing the insert in each of the two possible orientations with respect to the cloning vector. These were SJ2481 containing pSJ2481 and SJ2482 containing pSJ2482 (data not shown). Both clones produce α-amylase as detected on amylopectin plates at 60 °C. Fig. 4 schematically shows the various subconstructs and their phenotype, which represents the α-amylase activity.

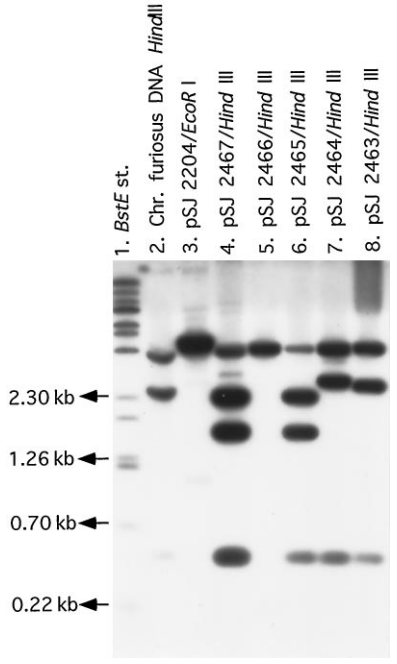

FIG. 3. **The Southern blot analysis of five clones (*lanes 4–8*).** Four clones (*lanes 6–8* and *4*) contain a common DNA region around 6.5 kb. That region is also found in *P. furiosus* chromosome. The same region (same *Hin*dIII pattern) is found in *P. woesei* DNA. Experimental details are under "Material and Methods."

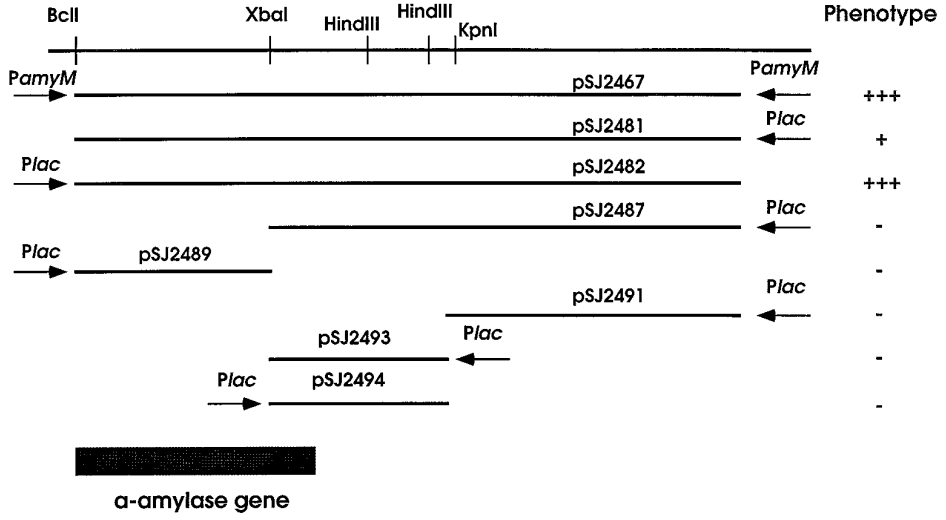

FIG. 4. **Restriction map of the cloned DNA; a number of subclones are indicated.** The phenotype of the clones plated on dyed amylopectin plates shows the α-amylase activity. At the *bottom*, the location of the α-amylase encoding gene as deduced from the DNA sequence is indicated.

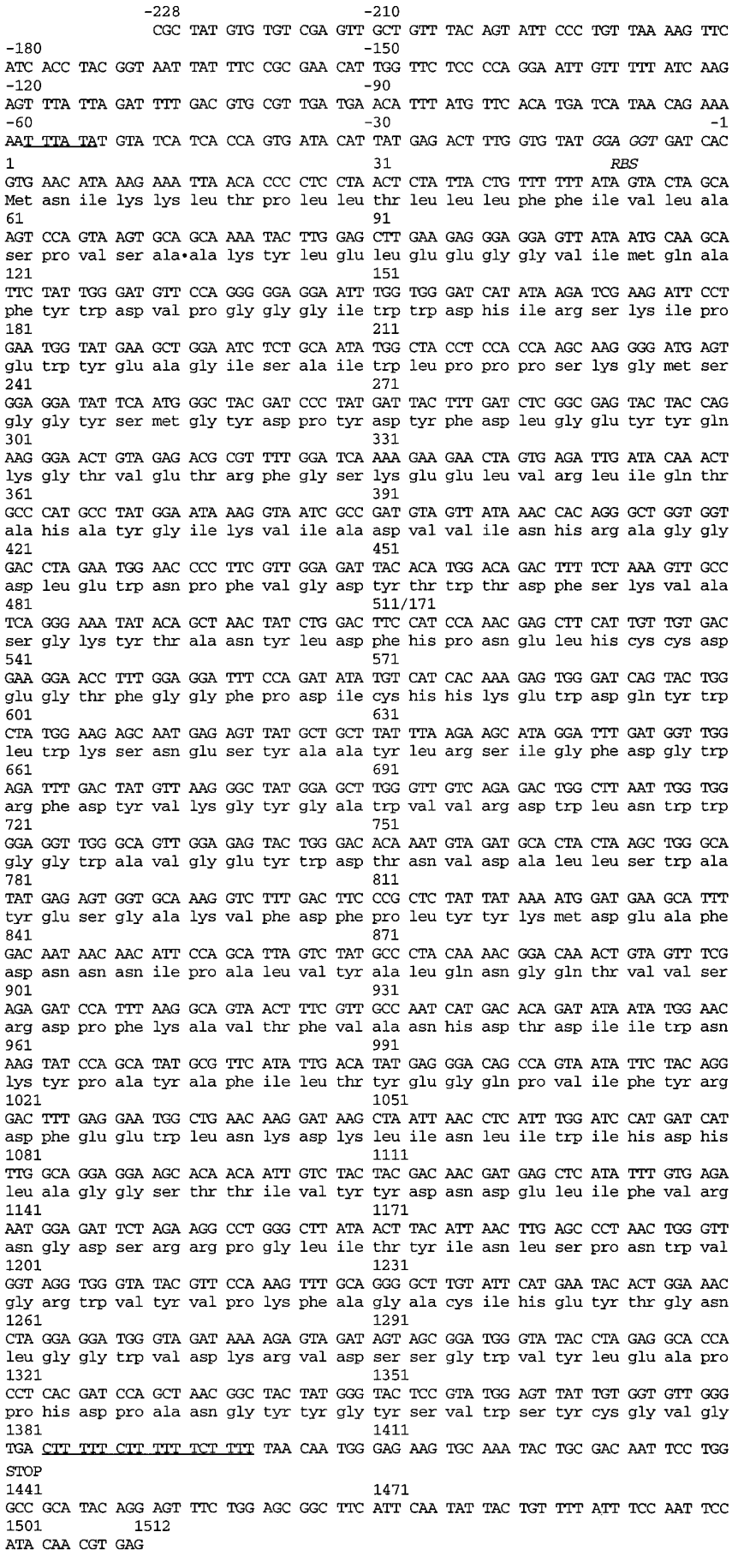

FIG. 5. **Nucleotide sequence and deduced amino acid sequence of the α-amylase gene from *P. furiosus*.** The nucleotide sequence between the −228 and 1512 sites is presented. The ribosome binding site GGAGGT (−6 to −9) 6 bases upstream of the start codon GTG (+1) is in *italics*. The promoter region containing a consensus BoxA sequence TTTATA (−53 to −58) is *underlined*. The site of the cleavage of signal peptide is between Ala^{25} and Ala^{26} and indicated by a *dot*. Immediately following the TGA stop codon there is a pyrimidine-rich sequence (which is between 1383 and 1401 and *underlined*) as found in other *Pyrococcus* sp. and archaeal genes. The α-amylase gene has been deposited in the GenBank™ under the accession number U96622.

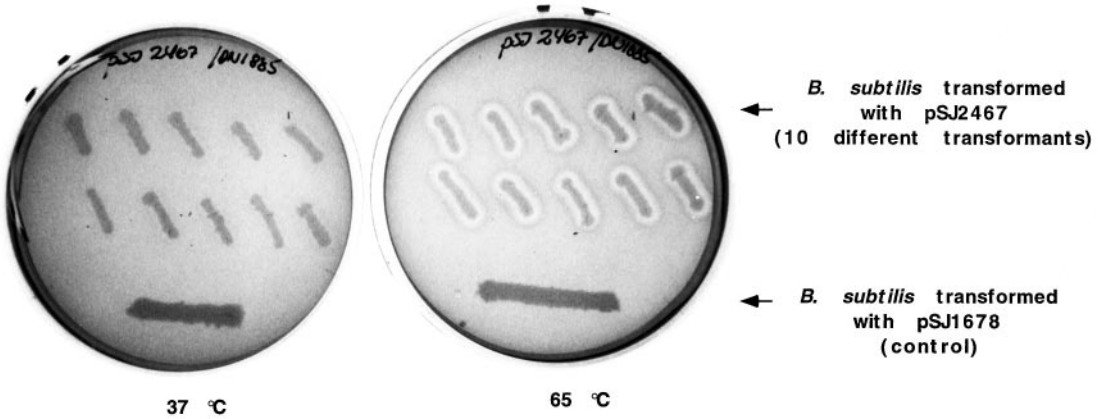

FIG. 6. **The amylase was expressed in *B. subtilis* by transforming the recombinant clone pSJ2467 obtained from the screening of the gene bank in *E. coli* into *B. subtilis*.** Ten tranformants and the control at 37 and 60 °C were plated.

Further subcloning was preformed from pSJ2481. pSJ2487 (see Fig. 4) was constructed by deletion of the 1-kb *Xba*I fragment from pSJ2481 and transformation of the religated plasmid into *E. coli* SJ2. The resulting transformants were not able to produce halos on LB plates containing dyed amylopectin, indicating that this deletion had removed a DNA region of importance for expression of an active amylase protein. The 1-kb *Xba*I fragment from pSJ2481 was inserted into *Xba*I-digested pUC19 to give pSJ2489 and pSJ2490 (identical), which were used for sequencing.

When a Southern blot (Fig. 3) prepared with digested genomic DNA from *P. furiosus* was probed with the ^{32}P-labeled pSJ2481, a 5.3-kb *Pst*I fragment, a 3.1-kb *Hin*dIII fragment, a 5.3-kb *Xho*I fragment, and two *Eco*RI fragments of 0.7 and 2.4 kb were found to specifically hybridize to the probe. The blot also shows that pSJ2463, pSJ2464, pSJ2465, and pSJ2467 contain a common DNA region (a *Hin*dIII fragment of approximately 0.5 kb is common to pSJ2463, pSJ2464, pSJ2465, pSJ2467, and chromosomal *P. furiosus* DNA). It also proves that the insert of pSJ2481 is derived from the chromosome of *P. furiosus* and that a homologous DNA region exists in the chromosome of *Pyrococcus woesei*. The chromosomal *P. woesei* DNA was isolated according to the method described in Ref. 15. Thus pSJ2481 hybridizes to exactly the same fragments in *Hin*dIII-digested *P. woesei* DNA as in *Hin*dIII-digested *P. furiosus* DNA. Both clones produced α-amylase as visualized by the appearence of clear halos on dyed amylopectin plates after incubation at 60 °C. The amylase-producing transformants look different when compared with transformants containing the pUC19 vector plasmid only. They form smaller and more translucent colonies.

The open reading frame corresponding to the α-amylase gene was localized by subcloning (the ability of individual subclones to produce α-amylase was assayed on plates containing dyed amylopectin) as outlined on Fig. 4. The 4.5 kb of the *P. furiosus* DNA insert cloned on pSJ2467 was sequenced on both strands using Sequenase™ and a combination of subclones and primer walking.

The DNA sequence of the α-amylase coding region, including the signal peptide coding region, is shown in Fig. 5. On the basis of the DNA sequence and N terminus amino acid sequence determination of the mature α-amylase, the amino acid sequences of the signal peptide and of the mature α-amylase have been deduced. The signal peptide is 25 amino acids long and is cleaved between Ala^{25} and Ala^{26}.

The DNA fragment containing the α-amylase gene encompasses 1740 nucleotides, with the initiation codon GTG at position +1 (Fig. 5). The 1380-base pair open reading frame encodes a single polypeptide with a molecular mass of 52,843 Da. This agrees well with the apparent molecular mass of the protein, determined by gel electrophoresis under denaturing conditions, of 54 kDa. Immediately upstream of the coding region is the sequence GAGGT identical to the putative ribosome-binding site of the glyceraldehyde-3-phosphate dehydrogenase gene of *P. woesei* (GAGGT) (21). A pyrimidine-rich region exists immediately downstream from the TAG termination codon in the α-amylase gene, like other archaebacterial sequences. A "box A" promoter region was also identified between −53 and −58. The G + C content of the α-amylase gene is 42.9%, slightly higher than the value reported for the total genome of 38% (7). As has been seen in other sequenced genes from extreme thermophiles, A and T are preferred bases in the third position of the codon (21).

Expression of the α-Amylase Gene in E. coli and B. subtilis—The plasmid pSJ1678 used for construction of the gene library is a shuttle vector able to replicate in both *E. coli* and *B. subtilis*. The *amy*M promoters, reading into the inserts cloned in this vector, are functional in *E. coli*, thus enhancing the chances that any gene cloned would be successfully expressed in this host. To examine the expression of the amylase activity in *B. subtilis*, pSJ2467 was therefore transformed into competent cells of DN1885 selecting for resistance to chloramphenicol (6 μg/ml) on LB plates containing dyed amylopectin. Few transformants were picked onto new plates with dyed amylopectin, and after incubation at 37 °C one plate was transferred to 65 °C, whereas the other was kept at 37 °C. Seven hours later a degradation of the amylopectin around the transformants with pSJ2467 was observed on the plates incubated at 65 °C as formation of a clear halo. No halo was formed at 37 °C for the control strain as shown in Fig. 6.

Biochemical Characterization—As shown in Fig. 7 (*A–C*), α-amylase from *P. furiosus* is active in a broad temperature range from 40 to 130 °C (Fig. 7*B*) and in a pH range from 3.5 to 8.0 (Fig. 7*A*) Maximal activity is measured at 100–105 °C and pH 4.5. Conditions for incubation of *P. furiosus* α-amylase for the determination of pH and temperature optimum are described under "Materials and Methods."

Beside the extremely high temperature optimum, the α-amylase shows remarkable thermal stability as well as stability against chemical denaturation. As depicted in Fig. 7*C*, incubation in a boiling water bath for 6 h causes a decrease in enzymatic activity of only 20%. Around 60% of the enzyme activity is still detectable after 120 °C for 1 h. Furthermore, after heating of the enzyme at 115 °C for 3 h, 35% of residual

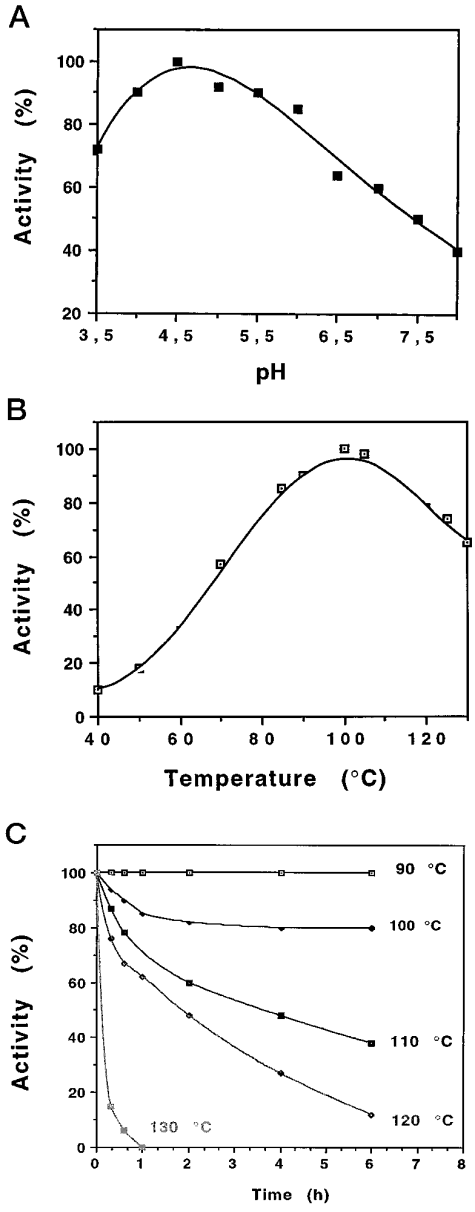

FIG. 7. *A*, activity pH dependency of α-amylase from *P. furiosus*. The extract containing the enzyme was incubated in water baths for measurements up to 90 °C or in glycerin bath for measurements between 90 and 130 °C. After various time intervals, as indicated on the plot, samples were withdrawn and analyzed for α-amylase activity as described under "Materials and Methods." *B*, temperature-dependent recombinant *P. furiosus* α-amylase activity. The reaction was carried out at the standard assay as described under "Materials and Methods" and at various temperatures as indicated on the plot. *C*, thermal stability of α-amylase from *P. furiosus*. The samples containing the enzyme were incubated at various temperatures (as indicated on the plot), and after various time intervals samples were withdrawn and the α-amylase activity was determined as described under "Materials and Methods."

activity was determined. The same sample was able to recover about 75% of its initial activity after 3 h of incubation (renaturation period) at room temperature. Some activity is still detectable after 30 min at 130 °C. On the other hand, the α-amylase is 55% active in the presence of 1.5 M urea or 61% in 0.3 M guanidine hydrochloride. The initial activity can be completely recovered after removing both denaturing agents by dialysis.

The addition of 5 mM of molybdenum, calcium, or magnesium ions did not influence α-amylase activity. A slight decrease of activity could be detected in the presence of cobalt, nickel, and iron ions, and complete inhibition was found when 5 mM of zinc or copper ions was added. Because EDTA did not show any effect, we can assume that the addition of metal ions is not required for enzymatic activity (data not shown).

TABLE I
Substrate specificity of α-amylase from P. furiosus

The activity of α-amylase from *P. furiosus* using various substrates is shown. The reaction was carried out at 90 °C for 30 min and at various substrate concentrations as indicated in the table. The values represent the enzyme activity as a percentage of the control.

Substrate	Concentration	α-Amylase activity
	%w/v	%
Starch	1	100
Amylose	0.2	84
Maltose	1	0
Maltotriose	1	0
Dextran	1	0
Maltodextran	1	14
Pullulan	0.5	0
Dextrin (white)	1	54
Xylan (Roth)	0.5	3
Xylan (Birchwood)	0.5	2
α-Cyclodextrin	1	0
β-Cyclodextrin	1	0
γ-Cyclodextrin	1	0
Starch (1%) + α-cyclodextrin (0.1%)		96
Starch (1%) + β-cyclodextrin (0.1%)		81
Starch (1%) + γ-cyclodextrin (0.1%)		87

Substrate Specificity—The partially purified extracellular α-amylase from *P. furiosus* hydrolyzes native starch, soluble starch, amylopectin, maltodextrin, and amylose as shown in Table I. Main products of starch degradation were oligosaccharides such as maltohexaose, maltopentaose, maltotetraose, maltotriose, and maltose. The enzyme degrades the α-1,4 glycosidic linkage in starch in a random fashion and can be designated as an α-amylase.

HPLC analysis has shown that the distribution of oligosaccharides seen in the chromatograms is typical of endo-amylase attack. The major oligosaccharides formed on prolonged hydrolysis are DP4, DP6, and DP7 (data not shown).

Comparison of α-Amylase from P. furiosus to the Other Known α-Amylases—The National Center for Biotechnology Information BLAST e-mail server was used to search the peptide sequence data bases (Brookhaven Protein Data Bank, SwissProt, Pacific Investment Research, Inc. and GenPept) for proteins homologous to the α-amylase protein sequence (19, 22). The α-amylase encoded by this open reading frame revealed homology to α-amylases and other starch-degrading enzymes from a variety of organisms including bacteria, insects, and plants, and it is classified in family 13 of the glycosyl hydrolases (3).

Nakajima and his colleagues (23) have identified four short primary sequence motifs, which also have been identified in amylolytic enzymes with other activities. These motives were also found in our α-amylase and indicated as regions I to IV in the alignment shown in Fig. 8*A*.

The highest scoring homologous sequences were all α-amylases, of both procaryotic and eucaryotic origin. A homology matrix based on the alignment is presented in Fig. 8*B*. It reveals that the *P. furiosus* α-amylase has about 47% homology to *Bacillus licheniformis* (Amy-Bacli) (24), 44% to *Salmonella typhimurium* (Amy2-Salty) (25), 39% to *Oryza sativa* (Amy1-Oryza) (26), and 34% to the *Pseudomonas stutzeri* (Amt4-Psest) (27) enzymes.

Comparison of the Subcellular and Extracellular α-Amylases from P. furiosus—Laderman *et al.* (5, 6) have also isolated and

A

Gene name	Region I	Region II	Region III	Region IV
Amt4_Psest	DVVPNH	GGFRFDFVRGY	VGELW	AVTFVDNHDT
Amy1_Orysa	DIVINH	DAWRLDFAKGY	VAEIW	ATTFVDNHDT
Amy2_Salty	DVVVNH	DGFRLDAVKHI	VAEYW	AVTLVANHDT
Amy_Bacli	DVVINH	DGFRLDAVKHI	VAEYW	AVTFVDNHDT
Amy1_Pyrfu	DVVINH	DGWRFDYVKGY	VGEYW	AVTFVANHDT
	+ + ++	+ +	+ + +	+ + + ++++

B

Gene name	Amt4_Psest	Amy1_Orysa	Amy2_Salty	Amy_Bacli	Amy1_Pyrfu
Amt4_Psest	100	49.1	31.2	35.2	34.1
Amy1_Orysa		100	36.2	38.1	39.2
Amy2_Salty			100	58.7	44.5
Amy_Bacli				100	47.1
Amy1_Pyrfu					100

FIG. 8. *A*, alignment of the conserved regions (I–IV) between our α-amylase (Amy1_Pyrfu) and the other four that have shown the highest homology scoring. *B*, table indicating the percentage of homology (identity) between our α-amylase and the other four highly related enzymes described under "Results."

TABLE II

Comparison of the two different α-amylases from P. furiosus

Comparison of some features of the extracellular α-amylase presented in this work and the subcellular α-amylase described by Laderman *et al.* (5, 6).

Properties	This work	Laderman *et al.*
Amino acids	461	649
Molecular mass (Da)	52875	76261
Cellular localization	extracellular	intracellular
Signal peptide	yes	no
Start codon	GTG	GTG
I–IV amylases conserved regions	yes	no
T-rich region at the 3′ terminus	yes	no

characterized a subcellular α-amylase from *P. furiosus*. We named our α-amylase "extracellular enzyme" to distinguish it from the subcellular enzyme. There are several differences between the two enzymes in terms of size, localization, and primary structure, which are summarized in Table II. Our extracellular α-amylase shows significant homology to the other α-amylase from a variety of organisms and has been classified in family 13 of glycosyl hydrolases, whereas the subcellular α-amylase shows strong homology only to α-amylase from *D. thermophilum*, and both have been included to an extra family 57 of glycosyl hydrolases.

DISCUSSION

We have isolated and sequenced the gene of a new extracellular α-amylase from the hyperthermophile archaeon *P. furiosus*. The gene was also expressed in *E. coli* and *B. subtilis* using a novel shuttle vector. The structure of the gene displays the typical characteristics of an archaeon gene with a typical ribosome binding site and a pyrimidine-rich region immediately downstream from the stop codon. The utilization of the GTG initiation codon, which is used relatively rarely, seems to be the rule in *Pyrococcus* sp. genes isolated so far.

The extracellular α-amylase enzyme is not very closely related to any other amylases of family 13 of glycosyl hydrolases. On the other hand it can be aligned to the other enzymes, and it has the conserved regions I-IV found in other amylases.

From the structural features of the extracellular α-amylase gene and the subcellular described by Laderman *et al.* (5, 6) as well as their primary structure comparison, it is clear that these are two completely different enzymes. This archaeon converts starch or glycogen to small linear and branched oligosaccharides, which can be transported most probably by "dextrin premease" into the cell. The presence of intracellular α-amylase indicates that further carbohydrate metabolism by *P. furiosus* is performed intracellularly.

The extracellular α-amylase from *P. furiosus* is one of a number of extremophilic enzymes that have been expressed in a mesophilic host in an active form. The fact that expression of amylase activity from a *Pyrococcus* α-amylase gene can be obtained in *B. subtilis* without any modification of the gene (for example replacement of the ribosome binding site) to allow more efficient initiation of translation is surprising. *B. subtilis* is quite restrictive in its acceptance of ribosome binding sites, and it is a frequent observation that cloned genes from non-Gram-positive organisms would not be expressed in *B. subtilis* without proper modification of their expression signals. To our knowledge, this expression of the *P. furiosus* α-amylase constitutes the first example of expression from an unmodified (or nonengineered) *Pyrococcus* gene in *Bacillus*.

The high thermostability of this pyrococcal α-amylase, its independence on metal ions, its unique substrate specificity, and its product pattern make this enzyme an interesting candidate for industrial application. It is therefore very important to employ genetic and fermentation techniques for the production of such enzymes on a large scale.

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