Secreted Expression of a Hyperthermophilic α -Amylase Gene from *Thermococcus* sp. HJ21 in *Bacillus subtilis*

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Key Words

Hyperthermophilic α -amylase · *Bacillus subtilis* · Promoter · Signal peptide · Secreted expression

Abstract

The hyperthermophilic α -amylase from *Thermococcus* sp. HJ21 possesses unique traits (Ca^{2+} -independent thermostability and optimal temperature of 95°C) that make it a great potential candidate for use in the food industry. However, this Archaea isolated from a deep-sea thermal vent requires strict control of culture conditions and produces only small amounts of α -amylase. To solve these problems, the α -amylase gene was cloned and expressed in *Bacillus subtilis*, which is an ideal food-grade host for heterologous protein expression. To express high levels of this α -amylase in *B. subtilis*, the promoters P_{grac} , P_{xylA} , P_{43} , and P_{hag} were used to construct four different expression vectors for testing. The vector containing the P_{xylA} promoter was found to have the highest transcriptional activity and produce the highest amylase activity (19.6 U/ml). To test the secretion efficiency of signal peptides in *B. subtilis*, three signal peptides were cloned and fused to the α -amylase gene (lacking its native signal peptide). The optimal signal peptide was S_{amyQ} , with a secretion efficiency of approximately 90%. These results indicate that the promoter P_{xylA} and signal peptide S_{amyQ} tested in this study may be useful for the expression and secretion of archaeal proteins in *B. subtilis*.

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Introduction

Bacillus subtilis is one of the most widely used hosts for the expression of heterologous proteins. In contrast to the well-known Gram-negative bacterium *Escherichia coli*, *B. subtilis* is generally recognized as safe and non-pathogenic [Earl et al., 2007]. The absence of an exterior membrane enables the direct export of proteins into the extracellular medium, simplifying the downstream processing of the proteins. Moreover, *B. subtilis* is regarded as an ideal expression system because of its well-understood genetics, well-established fermentation technology, and multiple regulators (e.g. promoters and signal peptides) that control expression and secretion [Fu et al., 2007]. About 60% of commercially available enzymes are currently produced by *Bacillus* species [Westers et al., 2004]. Therefore, scientists pay more attention to this organism with the aim to the commercial exploitation of *B. subtilis* for secreted heterologous proteins of interest.

The α -amylases (EC 3.2.1.1) are endo-acting enzymes that catalyze the hydrolysis of starch by cleaving α -1,4-glucosidic linkages, producing maltose and larger oligosaccharides. For centuries, amylases from plants, animals, and microbial sources have been used for brewing, processing starch, textile production, and the pharmaceutical

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Fig. 1. Map of the *E. coli*-*B. subtilis* shuttle vector pHT01-Amy. *P_{grac}* and *LacI* represent the IPTG-inducible promoter *P_{grac}* and *lacI* gene (lac repressor), respectively. *ColEI*, *CmR*, *AmpR*, and *Amy* represent the *E. coli* *ColEI* replicon, chloramphenicol-resistance marker, ampicillin-resistance marker, and coding sequence of α -amylase, respectively. Unique restriction sites are marked on the outside of the map.

industry [Hashim et al., 2004; Murakami et al., 2007, 2008; Pal and Khanum, 2010]. Because most of these industrial applications require thermostable enzymes, researchers have characterized amylases from various extremophilic microbes, including *Pyrococcus weesei*, *Pyrococcus furiosus*, *Thermococcus profundus*, *Thermococcus hydrothermalis*, and *Thermotoga maritima* [Vieille and Zeikus, 2001]. These hyperthermophilic amylases are tolerant, have a range of pH conditions and have a long shelf life [Chimata et al., 2011; Chou et al., 2010]. Among the most studied enzymes, the vast diversity of amylases makes them attractive for a variety of biotechnological applications [Wang et al., 2011].

We previously described the molecular and biochemical features of a hyperthermophilic α -amylase obtained from the new isolated anaerobic Archaea strain HJ21, which has been identified as *Thermococcus* [Wang et al., 2008]. Although its Ca^{2+} -independent thermostability and 95°C optimal temperature make this α -amylase a great potential candidate for industrial applications, the strict culture conditions and low enzyme production of *Thermococcus* HJ21 limit its further development. There-

fore, heterologous expression of this α -amylase in *B. subtilis* is desirable. In this study, the hyperthermophilic α -amylase gene was cloned and expressed in *B. subtilis*. To overproduce this enzyme and improve secretion efficiency, four promoters and three signal peptides were tested, and the transcription efficiency, secretion efficiency, and enzyme activity in the host were compared. Since genes and proteins from Archaea exhibit properties that differ from those of other microbes, this study furthers the understanding of heterologous protein expression in *B. subtilis*.

Results

Effect of Different Promoters on Amylase Transcription and Expression

To determine the expression efficiency of the four promoters, the recombinant plasmids pHT01-Amy, pHTP_{xyIA}-Amy, pHTP43-Amy, and pHTP_{hag}-Amy were transformed into *B. subtilis* DB104. Cells containing plasmids pHT01-Amy and pHTP_{xyIA}-Amy were grown to an optical density (OD) of 0.7–0.8 and induced with 0.1 mM IPTG and 0.5% (w/v) xylose, respectively. After a 24-hour incubation in LB medium, the amylase transcription level and enzyme activity of these strains were measured (fig. 1).

Results of real-time PCR (fig. 2a) showed that the transcriptional activities of pHTP_{xyIA}-Amy, pHTP43-Amy, and pHTP_{hag}-Amy were higher than that of the control plasmid, pHT01-Amy. In particular, the transcriptional activity of pHTP_{xyIA}-Amy suggested that it would be an excellent candidate to express this α -amylase in *B. subtilis*.

To further evaluate the efficiency of these promoters, α -amylase activity was measured. The plasmids pHTP_{xyIA}-Amy, pHTP43-Amy, and pHTP_{hag}-Amy produced higher levels of total amylase than pHT01-Amy (fig. 2b). After the 24-hour incubation, the amylase activity of cells harboring pHTP_{xyIA}-Amy and pHTP43-Amy was 3-fold (19.6 U/ml) and 1.8-fold (11.6 U/ml) higher, respectively, than that of cells harboring pHT01-Amy (6.6 U/ml).

The growth profiles of *B. subtilis* DB104 harboring pHT01-Amy, pHTP_{xyIA}-Amy, pHTP43-Amy, and pHTP_{hag}-Amy were approximately same (fig. 2c). This result demonstrates that the different transcriptional and enzyme activities of the strains were not a result of differences in growth.

Fig. 2. Transcriptional and enzyme activity of different promoters. **a** Real-time PCR analysis of amylase expression of cells harboring pHT01-Amy, pHTP_{xylA}-Amy, pHTP43-Amy, and pHTP_{hag}-Amy; 16S rDNA was used as the internal control. **b** Amylase activity of cells cultured in LB. The black diamond, black square, black triangle, and white circle represent the total amylase activity of *B. subtilis* DB104 cells harboring pHT01-Amy, pHTP_{xylA}-Amy, pHTP43-Amy, and pHTP_{hag}-Amy, respectively. **c** Growth curves of *B. subtilis* DB104 harboring plasmids with different promoters when cultured in LB. The black diamond, black square, black triangle, and white circle represent the OD₆₀₀ of *B. subtilis* DB104 harboring pHT01-Amy, pHTP_{xylA}-Amy, pHTP43-Amy, and pHTP_{hag}-Amy, respectively.

Effect of Different Signal Peptides on Amylase Transcription, Expression, and Secretion

Our results suggested that promoter P_{xylA} was an improvement over the control plasmid (pHT01-Amy) with regard to transcription activity and enzyme production after induction with 0.5% xylose. Therefore, we used P_{xylA} to drive expression of this amylase fused to different signal peptides in *B. subtilis*.

The four plasmids pHTP_{xylA}-Amy, pHTP_{xylA}-S_{amyQ}-Amy, pHTP_{xylA}-S_{Epr}-Amy, and pHTP_{xylA}-S_{YncM}-Amy were transformed into *B. subtilis* DB104. Figure 3a shows that the transcript level of α -amylase in cells harboring pHTP_{xylA}-S_{amyQ}-Amy was 20% higher than that of cells harboring the control plasmid pHTP_{xylA}-Amy; however, this difference was not significant. Without xylose induction, the transcriptional activity of pHTP_{xylA}-Amy was only 10% of xylose-induced transcription (fig. 3a). Total

amylase activity after the 24-hour incubation was 20.2 U/ml (pHTP_{xylA}-S_{amyQ}-Amy), 18.4 U/ml (pHTP_{xylA}-Amy), 17.2 U/ml (pHTP_{xylA}-S_{Epr}-Amy), and 18.9 U/ml (pHTP_{xylA}-S_{YncM}-Amy) (fig. 3b).

To determine the secretion efficiency of the three signal peptides, extracellular and intracellular amylase activities were analyzed separately after the 24-hour incubation. The results show that amylase activity in the culture medium accumulated to a maximum level of 18.2 U/ml (45.6 μ g/ml) with pHTP_{xylA}-S_{amyQ}-Amy, 11.1 U/ml (27.9 μ g/ml) with pHTP_{xylA}-Amy, 10.3 U/ml (25.8 μ g/ml) with pHTP_{xylA}-S_{Epr}-Amy and 12.7 U/ml (31.9 μ g/ml) with pHTP_{xylA}-S_{YncM}-Amy. The secretion efficiency of cells harboring pHTP_{xylA}-S_{amyQ}-Amy was the highest at 90% (fig. 3c). In contrast, the secretion efficiency associated with pHTP_{xylA}-S_{Epr}-Amy, pHTP_{xylA}-S_{YncM}-Amy, and pHTP_{xylA}-Amy (bearing the native signal peptide of α -amylase) was ap-

Fig. 3. Effect of different signal peptides on amylase transcription, protein expression, and secretion. **a** Real-time PCR analysis of amylase mRNA levels in cells harboring pHTP_{xylA}-Amy, pHTP_{xylA}-S_{Epr}-Amy, pHTP_{xylA}-S_{YncM}-Amy, and pHTP_{xylA}-S_{amyQ}-Amy, respectively; 16S rDNA was used as the internal control. pHTP_{xylA}-Amy* indicates results from pHTP_{xylA}-Amy without xylose induction. **b** Amylase activity after a 24-hour incubation. The black diamond, black square, black triangle, and black circle represent total amylase activity from *B. subtilis* DB104 harboring pHTP_{xylA}-Amy, pHTP_{xylA}-S_{Epr}-Amy, pHTP_{xylA}-S_{YncM}-Amy, and pHTP_{xylA}-S_{amyQ}-Amy, respectively. The white circle represents total amylase activity from *B. subtilis* DB104 harboring pHTP_{xylA}-Amy when cultured without xylose. **c** Secretion efficiency from cells harboring pHTP_{xylA}-Amy, pHTP_{xylA}-S_{Epr}-Amy, pHTP_{xylA}-S_{YncM}-Amy, and pHTP_{xylA}-S_{amyQ}-Amy, respectively.

proximately 60%. The growth curves of all strains were similar, demonstrating that differences in cell growth did not account for the differences in enzyme and transcriptional activity (online suppl. fig. S2; for all online suppl. material, see www.karger.com/doi/10.1159/000346215).

Discussion

Organisms from the domain Archaea are a highly diverse and abundant group that includes extremophiles, which thrive in harsh environments such as hot springs, salt lakes, and submarine volcanic habitats [Ding et al., 2008]. Proteins, especially enzymes, isolated from ex-

tremophiles are of particular interest because of their ability to remain stable and function in extreme conditions. However, the strict culture conditions and low enzyme production of these organisms are two main bottlenecks to industrial development. To overcome these drawbacks, *E. coli* is widely used for the heterologous protein expression [Dobrinski et al., 2010; Loke et al., 2000; Musfeldt and Schönheit, 2002; Ramli et al., 2011]. Unfortunately, recombinant proteins expressed by *E. coli* require complex purification steps to remove pathogenic substances before the proteins can be used in food, greatly increasing the cost of production. As a next-generation cell factory, *B. subtilis* may be a better host for the expression of these special enzymes.

Although numerous heterologous proteins have been produced by *B. subtilis*, few have been obtained from Archaea [Soto and Flores, 2003; Wu and Wong, 2002]. Therefore, *B. subtilis* expression systems for archaeal proteins require optimization. Attempts to improve the productivity of *B. subtilis* have historically focused on empirical approaches, such as the effects of various culture media on protein yields [Priest, 1989]. However, the introduction of the recombinant DNA technology has allowed a more directed intervention into the genetics of the production hosts [Jurgen et al., 2001]. To improve the expression and secretion of heterologous proteins, large-scale screening experiments have been performed to identify efficient promoters and signal peptides [Brockmeier et al., 2006; Phan et al., 2010].

Here we successfully expressed the α -amylase gene of *Thermococcus* sp. HJ21 strain in *B. subtilis* and improved its expression and secretion. To achieve high transcriptional and enzyme activities, four promoters were tested: (1) P_{grac} , a widely used IPTG-inducible promoter used in the commercial *E. coli*-*B. subtilis* shuttle vector pHT01; (2) P_{xylA} , a strong xylose-inducible promoter from *Bacillus megaterium* [Nguyen et al., 2005]; (3) P43, a well-characterized constitutive promoter from *B. subtilis* that is recognized by sigma A factor [Yue et al., 2009], and (4) P_{hag} , a constitutive promoter that is recognized by sigma D factor, which has a sequence that is distinct from other sigma factors [Chen and Helmann, 1995]. Our result suggests that the P_{xylA} promoter is more efficient in *B. subtilis* than the other promoters, with a transcription activity 3-fold higher when compared to the IPTG-inducible promoter as well as the highest enzyme activity (19.6 U/ml). To our surprise, the strong constitutive P43 promoter, which was predicted to be functional during exponential and stationary growth phases, produced only modest amounts of the recombinant protein [Kataoka et al., 2011]. Moreover, the transcriptional activity of the IPTG-inducible promoter P_{grac} was doubled when induced by lactose (data not shown). These results indicate that not all strong promoters are suitable for this α -amylase expression in *B. subtilis*. Many factors must be considered when designing an expression system for heterologous proteins, including promoter type, characteristics of the heterologous protein, promoter-gene interactions, and type of inducer. A strong, controllable promoter is essential to achieve high-level target gene expression in *B. subtilis* [Ming et al., 2010]. Our results indicate that the xylose-inducible promoter may be a useful control element in biotechnology applications.

Because most exported proteins are transported through the Sec pathway in *B. subtilis* [Yamane et al., 2004], we cloned two Sec-type signal peptides (Epr and YncM) that exhibit high secretion efficiency [Brockmeier et al., 2006]. The signal peptide AmyQ was chosen because of its ability to secrete high levels of the native protein (1–3 g/l) [Palva, 1982]. Although Epr and YncM have been shown to be efficient for both intracellular and extracellular protein secretion [Brockmeier et al., 2006], we found that they performed less well for the secretion of archaeal proteins. In contrast, cells harboring S_{amyQ} secreted 90% of the α -amylase into the growth medium, which is consistent with results of previous studies. These results are rational since it is still not possible so far to predict the effects of a specific signal peptide in combination with a selected target protein. To achieve efficient protein secretion, numerous attempts have been made to study the features of signal peptides including the recognition site, amino acid composition and net charge of the N-domain [Leloup et al., 1999; Zanen et al., 2005]. It became apparent that the secretion efficiency, especially for heterologous proteins, critically depends on the nature of the signal peptide [Caspers et al., 2010]. As a consequence, finding an optimal signal peptide that is based on screening and previous researches becomes time-consuming and relies mostly on luck. Our results show that the signal peptide S_{amyQ} not only exhibits a high secretory capacity for the protein of eubacteria [Phan et al., 2006], but is efficient for secreting heterologous protein of Archaea. This revived our hope of finding a pattern for efficient secretion of archaeal proteins.

In summary, this study tested various promoters and signal peptides to optimize the expression of heterologous recombinant proteins in *B. subtilis*. As previously demonstrated, a promoter or signal peptide that efficiently overexpresses one protein may not be able to produce high levels of other proteins. To determine the best promoters and signal peptides for the expression of archaeal proteins, more information about these regulators and heterologous proteins is needed.

Experimental Procedures

Bacterial Strains, Plasmids, Primers, and Growth Conditions

The *B. subtilis* strain DB104 (*his*, Δ *aprA3 nprR2 nprE18*), a generous gift from Professor Fuping Lu, Tianjin University of Science and Technology, was used as the host strain. *E. coli* DH5 α (Invitrogen) was used for plasmid construction and preparation. All bacterial strains were cultured in 250-ml shake flasks containing

50 ml of Luria-Bertani (LB) medium and shaking at 180 rpm, 37°C. Xylose and isopropyl β -D-1-thiogalactopyranoside (IPTG) were added as required. The following antibiotic concentrations were used for selection: 100 μ g/ml ampicillin (for *E. coli*) and 5 μ g/ml chloramphenicol (for *B. subtilis*). The plasmids and primers used in this study are listed in Supplemental Material.

DNA Manipulation Techniques

Recombinant DNA techniques involving plasmid purification, enzyme digestion and linkage, and transformation of *E. coli* and *B. subtilis* were performed according to Sambrook et al. [1989] and *Current Protocols in Molecular Biology* [Ausubel et al., 1993].

Construction of Plasmid Vectors

The *amy* gene encoding the thermophilic α -amylase was amplified from the plasmid pET32a-Amy, using primers P1 and P2. The 1.4-kb fragment was digested with *Xba*I and cloned into the *E. coli*-*B. subtilis* shuttle vector pHT01, which was digested with the same enzyme, resulting in pHT01-Amy (fig. 1). This plasmid has an IPTG-inducible promoter P_{grac} and served as a backbone into which three different promoters were inserted to drive expression of the α -amylase in *B. subtilis*. First, the xylose-inducible promoter P_{xylA} and the gene encoding the XylR repressor were cloned from the plasmid pHCMC04 using primer pair P3/P4. Two constitutive promoters (P_{43} and P_{hag}) were amplified from the chromosome of *B. subtilis* 168 using primer pairs P5/P6, and P7/P8, respectively. After digestion by *Sac*I and *Bam*HI, these amplified fragments were cloned into the corresponding sites of pHT01-Amy, resulting in pHTP_{xylA}-Amy, pHTP₄₃-Amy, and pHTP_{hag}-Amy.

Fusing Signal Peptides to the *amy* Gene by Overlap Polymerase Chain Reaction

The native signal peptide of this α -amylase was replaced by three different signal peptides by overlap polymerase chain reaction (PCR). The primers used in this experiment are shown in the supplemental file. The primer positions and mechanism of the fusion are depicted in online supplementary figure S1.

As predicted by the SignalP 3.0 Server [<http://www.cbs.dtu.dk/services/SignalP/>], a 75-bp signal peptide sequence was found in the *amy* open reading frame. The signal peptide of AmyQ from *Bacillus amyloliquefaciens* was cloned from plasmid pHT43 as an upstream fragment using primer pair P9/P10, and the *amy* gene lacking the signal peptide was amplified as a downstream fragment using primer pairs P11/P12. Then a mixture of the two fragments was used as a template and amplified by primers P9 and P12 to generate the fusion amylase S_{amyQ}-Amy. Using the same protocol, two Sec-type signal peptides (Epr and YncM) from *B. subtilis* were separately amplified from the chromosome of *B. subtilis* 168 and fused to the truncated *amy*, resulting in S_{Epr}-Amy and S_{YncM}-Amy (suppl. fig. S1). These three fusion genes were digested with *Xba*I and inserted separately into plasmid pHTP_{xylA}-Amy, yielding pHTP_{xylA}-S_{amyQ}-Amy, pHTP_{xylA}-S_{Epr}-Amy, and pHTP_{xylA}-S_{YncM}-Amy.

Isolation of Total RNA and Real-Time PCR

Bacterial cultures were harvested after a 24-hour incubation. Total RNA was isolated using the EZgene Bacterial RNA Kit (Biomiga R6616). The cDNA was synthesized using the M-MLV RT-PCR kit (Sangon SK2435), and real-time PCR was performed using the Real-Time PCR Kit (Sangon BS643). The *amy* gene was amplified with primer pair P19/P20, and *B. subtilis* 16S rDNA was

amplified as the internal control with primer pair P21/P22. The following PCR protocol was used: 10 min at 95°C, and then 40 cycles consisting of 15 s at 95°C, 20 s at 60°C, and 30 s at 72°C. Reactions were carried out in a real-time PCR detection system (Applied Biosystems StepOne).

Amylase Activity Assay

Cells were harvested by centrifugation (10,000 g, 10 min), and the extracellular amylase activity of the supernatant was determined. The cell pellet was resuspended in 50 mM phosphate-buffered saline (4.3 mM Na₂HPO₄·7H₂O, 137 mM NaCl, 1.4 mM KH₂PO₄, and 2.7 mM KCl (pH 7.2)) with 0.5% Triton X-100, and the cells were disrupted by sonication. Debris was removed by centrifugation at 8,000 g for 10 min at 4°C, and the intracellular amylase activity of the supernatant was determined. The protein content of each sample was determined according to a modification of the Bradford method using bovine serum albumin (Sigma Chemical Co.) as a standard. Secretion efficiency was defined as the ratio of extracellular amylase activity to total amylase activity (sum of extracellular and intracellular amylase activity).

A 10- μ l aliquot of enzyme was mixed with 190 μ l sodium acetate buffer (50 mM, pH 5.0) containing 1% soluble starch and incubated at 95°C for 15 min. One unit of amylase was defined as the amount of enzyme required to produce reducing sugars equivalent to 1 μ mol of glucose per minute. The concentration of reducing sugar was determined by the dinitrosalicylic acid method [Miller, 1959].

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