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High-temperature cultivation and 5′ mRNA optimization are key factors for the efficient overexpression of thermostable *Deinococcus geothermalis* purine nucleoside phosphorylase in *Escherichia coli*

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ABSTRACT

Overexpression of genes from thermophiles in *Escherichia coli* is an attractive approach towards the large-scale production of thermostable biocatalysts. However, various factors can challenge efficient heterologous protein expression – one example is the formation of stable 5′ mRNA secondary structures that can impede an efficient translation initiation.

In this work, we describe the expression optimization of purine nucleoside phosphorylase from the thermophilic microbe *Deinococcus geothermalis* in *E. coli*. Poor expression levels caused by stable secondary 5′ mRNA structure formation were addressed by two different approaches: (i) increasing the cultivation temperature above the range used typically for recombinant protein expression and (ii) optimizing the 5′ mRNA sequence for reduced secondary structures in the translation initiation region.

The increase of the cultivation temperature from 30 °C to 42 °C allowed a more than 10-fold increase of activity per cell and optimizing the 5′ mRNA gene sequence further increased the activity per cell 1.7-fold at 42 °C. Thus, the combination of high-temperature cultivation and 5′ sequence optimization is described as an effective approach to overcome poor expression levels resulting from stable secondary 5′ mRNA structure formation. We suggest that this method is especially suitable for improving the expression of proteins derived from thermophiles in *E. coli*.

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1. Introduction

Thermostable enzymes are attractive alternatives to their thermolabile homologues in many biocatalytic applications (Fernandes, 2010; Hildén et al., 2009; Littlechild et al., 2007; Synowiecki, 2010; Turner et al., 2007). For their industrial application production costs have to be low. As most thermophilic microorganisms are difficult to cultivate, the expression of genes from thermophiles in *Escherichia coli* is a convenient approach.

However, the heterologous biosynthesis of thermostable enzymes in the mesophilic host *E. coli* often requires measures to overcome poor expression levels or misfolding. For example poor expression resulting from codon bias between thermophilic donor and *E. coli* as host can be tackled by coexpression of rare tRNAs or codon optimization (Wang and Zhang, 2009). Disulfide bond formation, being a widespread feature stabilizing intracellular proteins from thermophiles, can pose another obstacle (Beeby et al., 2005; Cacciapuoti et al., 1999). Various strategies and new breakthroughs for handling disulfide bond formation in *E. coli* have been recently achieved (de Marco, 2009; Hatahet et al., 2010; Nguyen et al., 2011). Other factors including optimal folding temperature and the need of specific activation factors may play a pivotal role for the functional expression of genes from thermophiles: hence, in some cases the expression of thermophilic enzymes has been successful in the thermophilic host *Thermus thermophilus*, while the functional expression of the same enzymes in *E. coli* failed (Angelov et al., 2009; Hidalgo et al., 2004). The potential requirement of a higher cultivation temperature for the soluble expression of genes from thermophiles was also addressed by Koma et al. (2006). By cultivation at 43 °C or even 46 °C they successfully expressed genes from thermophiles in *E. coli* that were only hardly expressed in soluble form at 37 °C.

At the transcriptional level, the formation of secondary mRNA structures in the translation initiation region of mRNA has been recognized as an important determinant for poor recombinant protein expression levels in general (de Smit and van Duin, 1990; Griswold

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et al., 2003; Kudla et al., 2009). Accordingly, the optimization of the 5′ mRNA sequence has been objective for a number of scientific endeavors (Care et al., 2008; Cèbe and Geiser, 2006; Griswold et al., 2003; Jung et al., 2010; Khan et al., 2007; Na et al., 2010; Niemitalo et al., 2005; Sadaf et al., 2008). Recent findings make us believe that this factor has to be particularly addressed for the recombinant expression of genes from thermophiles in mesophilic hosts: a computational study of Gu et al. (2010) gives insight into natural variation of secondary mRNA structure stability within genes, genomes and among different species. By analyzing the genomes of 340 species the authors showed that stability of secondary mRNA structures within the coding sequence of genes is generally reduced near the start codon. Moreover they found that among prokaryotes this effect becomes weaker with higher optimal growth temperature. Since secondary mRNA structures are destabilized at higher temperature there seems to be less selection pressure on the reduction of secondary 5′ mRNA structure stability in thermophiles than in mesophiles. In practice this may implicate that the expression of genes from thermophiles in mesophilic hosts, at "unnatural" low temperature, is in particular hampered by translation inhibiting 5′ mRNA structures.

In this work we describe the expression optimization of a thermostable purine nucleoside phosphorylase (PNP) isolated from the moderate thermophilic bacterium *Deinococcus geothermalis* in *E. coli*. Nucleoside phosphorylases emerged as suitable biocatalysts for the preparation of modified nucleosides in chemo-enzymatic approaches, and thus are of industrial interest (Mikhailopulo, 2007; Prasad et al., 1999). As these biocatalytic processes often require elevated temperatures, thermostable nucleoside phosphorylases isolated from thermophilic origin (Taran et al., 2009; Visser et al., 2010) or stabilized by immobilization (Ubiali et al., 2004) are of special interest.

Initial attempts to express PNP from *D. geothermalis* (DgPNP) in *E. coli* resulted in a poor yield of recombinant protein. RNA folding predictions suggested stable 5′ mRNA structures as probable cause. Our approach to attenuate the stability of these translation inhibiting RNA structures and enhance the recombinant expression level of DgPNP involved: (i) optimization of the 5′ mRNA sequence through mostly synonymous codon substitutions and (ii) raising the cultivation temperature above the default range used usually for recombinant protein expression.

2. Materials and methods

2.1. Plasmid constructions

The *D. geothermalis* gene coding for purine nucleoside phosphorylase (GenBank accession no. ABF45792) was amplified (*Pfu* DNA polymerase, Fermentas; all oligonucleotides for cloning were purchased from TIB Molbiol). As template served genomic *D. geothermalis* DNA isolated before using standard protocols (Sambrook and Russell, 2001). The DgPNP gene was cloned via NdeI/HindIII digestion (FastDigest® restriction endonucleases, Fermentas, Vilnius, Lithuania) and ligation (T4 DNA Ligase, Roche) into a derivative of the expression vector pCTUT7 (Siurkus et al., 2010). The resulting expression vector will be reffered to as pDgPNP. DgPNP expression vectors with optimized 5′ mRNA sequence were generated in the same way, but here the template specific portions of the primers contained the new codon sequences (Table 1).

2.2. Secondary mRNA structure prediction and optimization

Temperature-dependent secondary-structural properties were predicted for mRNA sequences truncated after the 50th codon using programs RNAfold and RNAsubopt from the Vienna RNA Package version 1.8.4 (Mathews et al., 1999; Walter et al., 1994; Wuchty et al., 1999). The free energy of formation (ΔG_F) of base pairs in the translation initiation region of the mRNA was defined as $\Delta G_F = \Delta G'_C - G_C$, where G_C is the free energy of the constrained ensemble of secondary structures that contains no base pairs in the translation initiation region and G'_C is the free energy of its complement state with at least one base pair in that region. G'_C was calculated indirectly from G_C and G_U, the free energy of the unconstrained ensemble, both predicted using RNAfold, yielding the equation:

$$\Delta G_F = G_U - G_C + k_B T \ln\left(1 - e^{(G_U - G_C)/(k_B T)}\right),$$

where k_B is the Boltzmann constant and T is temperature. The translation initiation region was defined to include the AGGAGA ribosome-binding Shine–Dalgarno sequence and its downstream sequence until the end of the 6th codon. Based on translation inhibition experiments with small noncoding RNA's, secondary structures further downstream do not inhibit translation initiation (Bouvier et al., 2008).

The mRNA minimum free energy structure was predicted using RNAfold. The portion of the structure encompassing the translation initiation region was isolated. To evaluate the extent to which the substructure described the dynamical ensemble of secondary structures, a sample of 10^4 Boltzmann-weighted structures was drawn using RNAsubopt, and the mean number of identical base pairs between the Boltzmann ensemble and the substructure was estimated from the sample.

The secondary-structural properties of pDgPNP mRNA were analyzed as described above. The sequence was optimized using an automatic optimizer that suggested silent mutations to reduce inhibitory secondary structures (Niemitalo O., Neubauer P., and Juffer A.H., in preparation). For DgPNP, the theoretical optimization results were unsatisfactory, for which reason a rational approach was taken to reduce by non-silent mutations the stability of inhibitory secondary structures. The second codon GTG was deleted. Then, a codon substitution valine2 → isoleucine: GTG → ATT was made, resulting in the variant DgPNP1. To this sequence, the optimizer could suggest further silent codon substitutions: Ala3: *GCG* → *GCC*, Arg4: CGT → CGA, Pro6: CCG → CCC. Additionally, a rare codon was eliminated by Arg8: AGG → CGT. The substitutions resulted in the variant DgPNP2.

2.3. Bacterial strains and cultivation conditions

E. coli TOP10 cells (Invitrogen) were used for cloning and harboring generated expression plasmids; *E. coli* BL21 (Novagen) served as expression strain. All transformations were performed by electroporation (electroporator 2510, Eppendorf) of purified plasmids (Invisorb® Spin Plasmid kit, Invitek, Berlin, Germany) following a standard protocol (Sambrook and Russell, 2001). Cultivations were performed in a shaking incubator with 2.5 cm shaking orbit (Kühner, Basel, Schweiz), at 250 rpm for expression in 10 ml Deep well plates (HJ-Bioanalytik, Mönchengladbach, Germany). Cells from LB agar plates grown overnight were used to inoculate LB medium to an initial OD_{600} value of 0.2. Protein expression was induced by addition of 100 μM or 1 mM IPTG after 2 h (cultivations performed at 42 °C), 2 1/2 h (37 °C) and 3 h (30 °C). Cells were harvested 3 h after induction.

2.4. Protein expression analysis and activity assay

After harvest (centrifugation at 16,000 × *g* for 5 min, 4 °C) cells were lysed with BugBuster® Protein Extraction Reagent (Novagen) according to the manufacturer's instructions. Soluble and insoluble protein fractions were separated by centrifugation at 20,000 × *g*

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