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# Comparison of two type IV hyperthermophilic adenylyl cyclases characterizations from the archaeon *Pyrococcus furiosus*

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ABSTRACT

In this paper, two genes that encoded two soluble type IV adenylyl cyclases (AC) from the hyperthermophilic archaeon *Pyrococcus furiosus* (PFAC I and PFAC II) were cloned and expressed in *Escherichia coli* (*E. coli*) BL21 (DE3). Amino acid sequence analysis of the two enzymes showed 29% homology. PFAC I and PFAC II were both $Mn^{2+}$ activated enzyme. They were purified by His-trap chromatography and had a specific activity of $3.1 \times 10^3$ U/mg at pH 10.0, 95 °C (PFAC I) and $2.0 \times 10^3$ U/mg at pH 11.0, 95 °C (PFAC II), respectively. The $K_m$ and $k_{cat}$ of PFAC I was 1.38 mM and 1.11 $s^{-1}$. The $K_m$ and $k_{cat}$ of PFAC II was 1.44 mM and 0.80 $s^{-1}$. The thermostability of PFAC I and PFAC II were higher than the soluble type IV adenylyl cyclases from *Yersinia pestis* (YpAC-IV). All of the properties suggested that these two adenylyl cyclases may be useful for the industrial producing of cyclic adenosine 3′,5′-monophosphate (cAMP).



## 1. Introduction

Cyclic adenosine 3′,5′-monophosphate (cAMP) is the most important nucleotide signal molecular which is used for intracellular communication, just like the effects of hormones adrenaline and glucagon which cannot pass through the membrane of cells [1,2]. The cAMP and its associated kinases have been found to be related to several biochemical processes in eukaryotes, including the regulation of lipid metabolism, glycogen and sugar. In bacteria, the cAMP has also been found to control a variety of processes which are from utilization of alternative sugars to motility and virulence [3,4]. Lots of researches have demonstrated an aberrant activation of cAMP-controlled genes and a deregulation of cAMP pathways are related to some kind of carcinoma [5–7]. In the pharmaceutical industry, the cAMP is an important substrate for the synthesis of used widely calcium dibutyryladenosine cyclophosphate (DCM) which has significantly therapeutic effect on heart failures [8].

As known, adenylyl cyclase (EC: 4.6.1.1, Fig. 1) is a phosphorus–oxygen lyase enzyme that catalyzes the biosynthesis of cAMP with elimination of diphosphate from adenosine 5′-triphosphate (ATP) (Fig. 2). Six different types of ACs (from AC-I to AC-VI) representing distinct gene families with unrelated topologies have been identified to provide key signaling and different regulatory functions [9–11], in various organisms [12,13] (Table 1). For example, AC-III have been discovered in mammalian and mycobacterial with transmembrane and soluble isoform [14,15].

Type IV AC (AC-IV) are found in the bacterial species *Yersinia pestis* [24]. Reported soluble AC-IV that are the smallest naturally occurring ones are encoded by approximate 180 residues. Genes homologous to *Y. pestis* AC-IV (YpAC-IV) are found in many archaea; for example, there are two similar genes in *Pyrococcus furiosus*: AAL80987.1 (with protein structure 1YEM, encoding PFAC I) is 23% identical with YpAC-IV, while AAL81983.1 (encoding PFAC II) is 32% [27].

*P. furiosus* is a hyperthermophilic species of archaea which is notable for having an optimum growth temperature at 100 °C [28]. So thermophilic enzymes from *P. furiosus* in which application interest is related to the fact that performing biotechnologically related processes at higher temperatures is often advantageous have been researched widely [29,30]. Although the YpAC-IV have been studied comprehensively [31], the characterizations of PFAC I and PFAC II being homologues with YpAC-IV have not been reported yet. To an excellent and thermostable biocatalyst for biosynthesis of cAMP, the aim of this paper is to clone, overexpress and characterize two type IV hyperthermophilic adenylyl cyclases from *P. furiosus*.

## 2. Materials and methods

### 2.1. Materials

The adenosine triphosphate (ATP) and cyclic adenosine 3′,5′-monophosphate (cAMP) were purchased from Sigma Co. The

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**Table 1**
Representatives of the six classes of adenylyl cyclase enzymes.

| Class | Source | MM (kDa) | Activator | pH | Soluble? |
|---|---|---|---|---|---|
| I | *Y. pestis* | 97 | $Ca^{2+}$ | 6.0–8.0 | Soluble |
| | *E. coli* [16] | 97 | $Mg^{2+}$ | 8.5–9.0 | Soluble |
| II | *P. aeruginosa* [17] | 43 | $Ca^{2+}$ | 8.5 | Soluble |
| | *B. anthracis* [18] | 92 | $Ca^{2+}$ | 7.4 | [a] |
| | *B. pertussis* [19] | 188 | $Ca^{2+}$ | ? | [a] |
| III | Mammalian (1 isoform) [20] | 187 | $Ca^{2+}$ | 7.4 | Soluble |
| | Mammalian (9 isoforms) [21] | 120–150 | $Mn^{2+}/Mg^{2+}$ | 7.4 | TM |
| | *M. tuberculosis* (Rv1264) [22] | 45 | $Mn^{2+}$ | 5.0–8.0 | Soluble |
| IV | *P. furiosus* | 20 | $Mn^{2+}$[b] | 10.0–11.0[b] | Soluble |
| | *Y. pestis* [23] | 20 | $Mn^{2+}$ | 10.4 | Soluble |
| V | *P. ruminicola* [24] | ~67 | ? | ? | ? |
| VI | *R. etli* [25] | ~38 | Mg2+ | ? | ? |

[a] These toxin ACs are initially soluble, then become anchored in the host-cell membranes [26].
[b] In this paper? No reported yet.

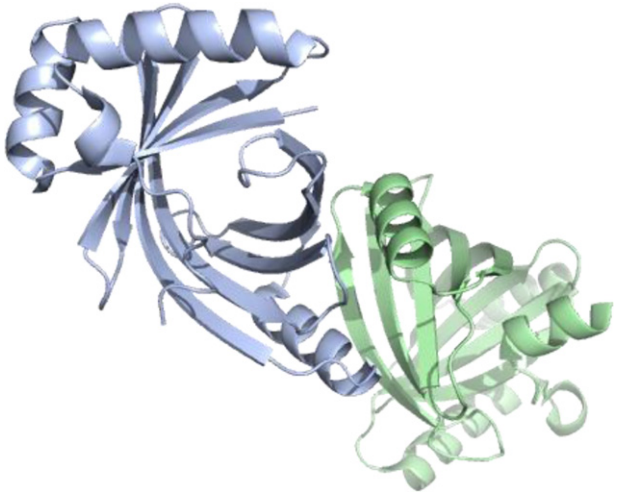

**Fig. 1.** The structure of adenylyl cyclase IV. The structure information is from 1YEM (PDB) which is adenylate cyclase from *P. furiosus*.

genome of *P. furiosus* was denoted by Jian Wu Ph.D (Biofuels Institute of Jiangsu University, China). All of the restriction enzymes, genomic extraction kits, PCR kits and plasmid extraction kits were purchased from Takara Co. The plasmid pET24a_(+), nickel-nitrilotriacetic acid (Ni-NTA) His-Bind Purification Kit, *E. coli* BL21(DE3) were purchased from Invitrogen Co. The bicinchoninic acid (BCA) kits, ethylenediaminetetraacetic acid (EDTA) and other reagents were purchased from Dingguo Co.

### 2.2. Cloning and expression

The *E. coli* BL21 (DE3), plasmid pET24a_(+) and the genomic DNA of *P. furiosus* (ATCC: 43587) were used as host cells, vectors and the source of adenylyl cyclase (AC) gene in this experiment. The genomic DNA isolated from *P. furiosus* was used as template of amplification of the gene encoding the adenylyl cyclase. Based on the DNA sequence of *P. furiosus* adenylyl cyclase I (PFAC I) and *P. furiosus* adenylyl cyclase II (PFAC II) gene (AE009950.1: AAL80987.1, AAL81983.1.), forward primers PF1 (5′-AAGCTTATGGAAGTTGAAATA-3′), PF2 (5′-AAGCTTATGAAGAGAGGCTGG-3′) and reverse primers PR1 (5′-GCGGCCGCTCATGAAGAGCGTCC-3′), PR2 (5′-GCGGCCGCTTAAGTATG TCGAGA-3′) contained Hind III and Not I (underlined sequences) restriction sites, respectively were synthesized by Anygene Co. (China). Amplification was performed using 2.5 units of DNA polymerase in a 100 μl reaction mixture of PCR reaction buffer, 1 μM each primer (PF1, PF2, PR1 and PR2) 0.2 mM each dNTP, 0.2 mg *P. furiosus* genomic DNA. Prior to cycling, the reaction mixture was heated to 95 °C for 1 min, followed by 30 cycles of 94 °C for 40 s, 55 °C for 1 min, 72 °C for 40 s, and a final extension at 72 °C for 5 min. The DNA fragments (516 bp and 576 bp) amplified by PCR were respectively digested with Hind III and Not I and ligated into the expression vector pET24a_(+) to finally create expression plasmids: pET24a-PFAC I and pET24a-PFAC II. The nucleotide sequences of the PCR-generated genes were determined by Sangon Co. The initial similarity search was carried out with the BLAST on the National Center for Biotechnology Information (NCBI).

Prepared *Escherichia coli* BL21 (DE3) cell containing pET24a-PFAC I and *E. coli* cell containing pET24a-PFAC II was respectively cultivated in a 1000 ml flask containing 250 ml of Luria–Bertani (LB) medium containing 1% (wt/vol) bacto-tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol) NaCl and 50 μg/ml kanamycin at 37 °C with shaking at 200 rpm. After the optimal density of bacteria reached 0.60 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.05 mM to induce the expression of adenylyl cyclase, and then the culture was incubated with shaking at 120 rpm at 30 °C for 16 h.

### 2.3. Preparation and purification of enzymes

The cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C and resuspended in lysis buffer, (50 mM Tris–HCl buffer, pH 7.0, containing 300 mM NaCl, and 10 mM imidazole) followed by disruption by ultra-sonication (400 W). The whole process of ultra-sonication lasted for 100 cycles. Each cycle included 4 s of

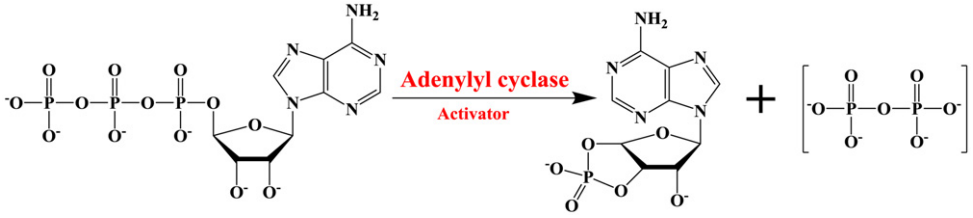


**Fig. 2.** Biosynthesis of cAMP from ATP by adenylate cyclase.